STUDIES ON NEWLY DISCOVERED OESTROGEN DERIVATIVES FROM URINE

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SECTION I. GENERAL INTRODUCTION

Work on the metabolism of oestrogens has been proceeding at an increasing rate in recent years. The considerable advances made have been achieved to a large extent by the use of new techniques, such as the employment of isotopically labelled compounds and the extensive application of chromatographic methods. In the identification of minor oestrogen metabolites the classical colour reactions as well as the more recent micro-techniques of infrared spectrometry have been of immense aid.

The biosynthesis of oestrogens can be more conveniently studied with the advent of labelled steroidal compounds. Thus, the *in vitro* conversion of radioactive testosterone by human ovarian tissue to oestrogens has been demonstrated by Baggett, Engel, Savard & Dorfman (1956) and Wotiz, Davies, Lemon & Gut (1956). These are complementary to the elegant studies of Meyer (1955) who, using classical techniques, showed the formation of oestrone from 19-hydroxy-androst-4-ene-3:17-dione by endocrine tissues. One of the/

the metabolic pathways of cholesterol in the human has been reported to lead to oestrone (Werbin, Plotz, LeRoy & Davies, 1957). Finally, the biosynthesis of oestrone, equilin and equilenin in the pregnant mare from administered $[1-^{14}C]$ acetate must be mentioned (for example, see review by Heard et al., 1956). It was found that the oestrone and the ring B unsaturated oestrogens isolated from the urine had different specific activities, and the conclusion was therefore made that the latter might have been derived from a precursor different from that of oestrone. Indeed, a postulate has been put forward that the formation of equilenin might involve the in vivo condensation of an ethyltoluene derivative with a C_{10} isoprenoid unit (Dorfman, 1956).

Some information on the catabolic fate of the primary hormone may be obtained by the isolation of its metabolites from urine. In view of the greatly elevated endogenous production of oestrogen during pregnancy, late pregnancy urine seems to be the ideal biological material for such/

such a study. In this Department the isolation of 16-*epi*oestriol from pregnancy urine was achieved in 1955 (Marrian & Bauld, 1955). In a subsequent search for the hypothetical metabolic precursor of oestriol and 16-*epi*oestriol, namely, 16-oxooestradiol-17β, Watson & Marrian (1955) detected a fifth Kober chromogen (KC-5) in the phenolic-ketonic extracts from pregnancy urine which they provisionally identified as the sought-for metabolite on partition chromatographic and countercurrent distribution evidence.

However, when KC-5 was isolated from the same source as a crystalline solid, it became apparent that the main component in KC-5 might be 16α-hydroxyoestrone contaminated with some 16-oxooestradiol-17β, the latter probably being produced artifactually from the former by rearrangement during the phenolic separation (Marrian, Watson & Panattoni, 1957). 16α-Hydroxyoestrone was eventually isolated from KC-5 and, in view of the ease with which it was found to rearrange to 16-oxooestradiol-17β in aqueous/

aqueous alkaline solution, it was concluded that pregnancy urine extracts probably contain no 16-oxooestradiol-17β other than that formed artifactually (Marrian, Loke, Watson & Panattoni, 1957; Section IIIA).

At this stage a sixth Kober chromogen (KC-6) more 'polar' than KC-5 was detected in pregnancy urine extracts (Section IV). It was considered likely that this might be 16β-hydroxyoestrone - the hypothetical metabolic precursor of 16-*epi*oestriol, just as 16α-hydroxyoestrone is of oestriol. It may be added that the metabolic relationship between 16α-hydroxyoestrone and oestriol has since been amply substantiated by the *in vivo* work in man of Brown & Marrian (1957). Thus, while efforts were being directed towards the isolation of KC-6, an attempt was concomitantly made to prepare 16β-hydroxyoestrone for subsequent identification purposes (Section IIIB).

With the availability of 16β-hydroxyoestrone, it became clear that KC-6 was not identical with it. From concentrates of KC-6 two crystalline oestrogen metabolites were subsequently isolated. One of these has been conclusively identified as 18-/

18-hydroxyoestrone, while the other also appears to be a mono-hydroxylated derivative of oestrone.

16β-Hydroxyoestrone undergoes rearrangement to 16-oxooestradiol-17β in both acid and alkaline solution. In this respect it is more labile than its 16α-epimer. Indeed, it was found by Layne & Marrian (work to be published) that ten minutes' exposure to alkaline solution sufficed for its complete conversion to 16-oxooestradiol-17β. However, by avoiding the alkaline fractionation (i.e. the phenolic separation) these workers have been able to detect 16β-hydroxyoestrone in neutral phenolic extracts from pregnancy urine. Its isolation from pregnancy urine together with 16-oxooestradiol-17β and 16α-hydroxyoestrone has recently been accomplished by these workers. It is to be noted that the isolation of 16-oxooestradiol-17β by a process which, as far as could be determined, could not have led to the artifactual formation of the compound from either 16α-hydroxyoestrone or 16β-hydroxyoestrone, establishes it as a normal metabolite and thus renders invalid the earlier conclusion of Marrian, Loke, Watson & Panattoni/

Panattoni (1957).

Of the numerous studies on the metabolism of administered oestrogen, that of Levitz, Spitzer & Twombly (1956) deserves special mention. It was found that $[16\text{-}^{14}C]$ oestradiol-17β was converted partly to $[16\text{-}^{14}C]$ 16-oxo-oestradiol-17β in the human. At one time there was some controversy on the presence of 16-oxo-oestradiol-17β in urine, for the possibility existed that it might be artifactually formed from 16α-hydroxyoestrone (cf. above). However, another metabolic pathway to 16-oxooestradiol-17β was revealed in a subsequent study from the same laboratory (Levitz <u>et al.</u>, 1957). $[16\text{-}^{14}C]$ Oestriol was found to give rise to $[16\text{-}^{14}C]$ 16-oxooestradiol-17β and $[16\text{-}^{14}C]$ 16-<u>epi</u>oestriol, which were identified as such after isolation by the reverse isotope dilution technique from the urine of the treated subject. These findings show clearly that oestriol undergoes partial oxidation at C-16 in the body to form 16-oxo-oestradiol-17β and that the latter compound is metabolically reduced to 16-<u>epi</u>oestriol. However, it is possible that the metabolic reduction of 16β-/

16β-hydroxyoestrone may still be the predominant route to 16-*epi*oestriol.

The isolation of 18-hydroxyoestrone from pregnancy urine is of interest, since it indicates that metabolic transformation is not necessarily confined to positions 16 and 17 of the oestrogen nucleus. As will be seen in Section V, hydroxylations at positions 11β and, probably, 12α of oestrone also occur on incubation of the substrate with ox adrenal homogenates. It may be added that the *in vitro* conversion of [16-^{14}C] oestradiol-17β to the 6β-hydroxy and the 6-oxo derivatives by rat liver microsomes has been reported recently (Mueller & Rumney, 1957). It remains to be seen which of these hydroxylations has its counterpart in the human species.

Besides conjugation, the metabolic transformations so far demonstrated in the oestrogen series are hydroxylation and the interconversion of a secondary alcoholic hydroxyl and the corresponding ketone. However, a novel biochemical reaction has been revealed by the recent isolation of [16-^{14}C] 2-methoxyoestrone from the urine of human subjects treated with [16-^{14}C] oestradiol/

oestradiol-17β or oestrone (Kraychy & Gallagher, 1957a,b). This finding was soon confirmed by Engel, Baggett & Carter (1957), while in the course of our studies on urinary oestrogen metabolites the same compound has now been isolated from the urine of pregnant women (Section VI; Loke & Marrian, 1958). Moreover, two other 2-methoxy derivatives, tentatively identified as 2-methoxy-16α-hydroxyoestrone and 2-methoxy-16-oxooestradiol-17β, have been detected in pregnancy urine (Section VII).

Since the formation of these 2-methoxy derivatives presumably involves prior oxygenation at position 2, these findings may be considered as providing tangible support to the early belief that one of the inactivation processes undergone by oestrogens in mammalian systems might involve the rupture of ring A <u>via</u> an °-hydroxylated intermediate (cf. Pearlman, 1948). In view of this, the field of research opened up by the discovery of 2-methoxyoestrone by Kraychy & Gallagher promises to be of great interest.

Another recent finding which may have extensive implications is the isolation of equilenin/

equilenin from a male adrenal tumour by Salhanick & Berliner (1957). In spite of the observations made in the pregnant mare (cf. Heard _et al._, 1956) it is possible that the equilenin is derived from oestrone by desaturation of ring B. Moreover, the ability of the human organism to effect desaturation is already indicated, for instance, by the isolation of androst-16-ene-3α-ol from women's urine (Brooksbank & Haslewood, 1952). A search for unsaturated oestrogen derivatives, e.g. 1,3,5(10),16-oestratetraen-3-ol, in urine may prove fruitful.

It is evident from this brief survey that much qualitative information has been obtained on oestrogen metabolism during the last five years. Insofar as the balance between health and disease is dependent on the quantitative rather than the qualitative aspects of oestrogen metabolism, it is therefore hoped that these findings will soon be followed up by quantitative investigations.

II. INVESTIGATION TOWARDS THE DEVELOPMENT OF A METHOD FOR THE QUANTITATIVE ESTIMATION OF 16-OXOOESTRADIOL-17β (KC-5)

1. Introduction

With the isolation of 16-*epi*oestriol from pregnancy urine (Marrian & Bauld, 1955) and the subsequent detection of a fifth Kober chromogen in pregnancy urine (Watson & Marrian, 1955) which was indistinguishable from 16-oxo-oestradiol-17β in its partition behaviour in several solvent systems, it became evident that future determinations of oestrogens in urine and other biological fluids must include these two new metabolites. While 16-*epi*oestriol is not of much quantitative significance - its concentration in menstrual cycle urines being roughly equal to that of oestradiol-17β - preliminary data obtained for KC-5 indicated that this common precursor of both 16-*epi*oestriol and oestriol is metabolically important from the quantitative standpoint.

Moreover, in recent years biological and clinical studies have led to the recognition that oestriol is not simply a weak oestrogen but a substance with interesting individual activities.

For one thing it is generally believed that oestriol has a unique physiological role to play during late pregnancy, though how this is performed is still obscure. Recently Puck & Hubner (1956) reported that oestriol displayed remarkable activities on the cervix of the uterus and on the vagina in the guinea pig and the rabbit, and they assume that oestriol might very well have a pronounced significance especially in preparing the cervix and the vagina for parturition. In the clinical field Bauld, Milne and Givner (1957) have observed that the excretion of oestriol in patients with myocardial infarction was considerably higher than that in normal patients. McBride (1957) using Brown's method (1955) noted that in all normal postmenopausal women studied the excretion of oestriol exceeded those of oestrone and oestradiol-17β together. Moreover, in some of the urines there was only oestriol with practically no oestrone or oestradiol. Thus, it would be interesting to see if the pattern of urinary KC-5 excretion resembles that of oestriol.

Among the chemical methods available at present for the estimation of urinary oestrogens, those of Brown and Bauld (1956) have been shown to be satisfactory in regard to accuracy, precision, sensitivity and specificity. However, these were developed at a time when only three oestrogens, namely, oestrone, oestradiol and oestriol, were known.

While it would not be difficult to modify either of these methods for the determination of 16-epioestriol - indeed, by modifying the method of Bauld (1953) for oestriol, Watson & Marrian (1956) have carried out some determinations of 16-epioestriol in menstrual cycle urines - the determination of another oestrogen metabolite having a 'polarity' intermediate between oestrone and oestradiol, on the one hand, and 16-epioestriol and oestriol on the other, must necessarily involve some radical changes. For instance, the aqueous benzene partition method used for the separation of these two groups of oestrogens would not be suitable in the presence of 16-oxooestradiol-17β. Hence, in developing a method for the detection of 16-oxooestradiol-17β in pregnancy urine, Watson & Marrian (1955) omitted this step and instead separated the five oestrogens into two fractions by means of the Girard reaction. Subsequent partition chromatography was therefore restricted to two columns.

It is the finding that enzyme hydrolysis yielded more KC-5 than acid hydrolysis, and the subsequent demonstration that 16-oxooestradiol-17β is labile to acid hydrolysis in the presence of urine, which pointed to the need for a most radical modification in both isolation and analytical work. It seems that enzymic hydrolysis is the only alternative other than the estimation of the oestrogens in the form of their conjugates.

The method developed by Watson & Marrian consists of the following steps:-

(a) acid or enzymic hydrolysis of urine
(b) ether extraction of the liberated oestrogens
(c) removal of the acidic fraction from the extract
(d) separation of the neutrals-phenols into ketonic and non-ketonic fractions
(e) separation of the ketonic neutrals and phenols
(f) partition chromatography of the ketonic phenols and colorimetric estimation.

Recoveries with pure 16-oxooestradiol-17β added to urine were not as satisfactory as one would have wished, but this method proved its worth in the detection of KC-5 in pregnancy urine. Thus, a logical forward step in the development of a quantitative method for the estimation of 16-oxooestradiol in urine would be to study the method more thoroughly.

This section of the thesis describes several findings which led to a modification of the original method of Watson & Marrian. With this modified procedure some recovery experiments have been carried out with satisfactory results.

2. General Methods

(a) Acid hydrolysis of urine

This was carried out by heating the urine to boiling, adding 15 vols. % concentrated HCl and continuing the boiling under reflux for 60 min.

(b) Preparation of soluble β-glucuronidase from *Patella vulgata*

For quantitative work a water-soluble preparation was prepared by extracting the crude acetone-dried powder obtained from *Patella vulgata* by the method of Dodgson & Spencer (1953) with water and reprecipitating the water-soluble glucuronidase from this solution by the addition of about 8 times its volume of acetone. The resultant powder was collected, dried and subsequently standardized for β-glucuronidase activity with phenolphthalein glucuronide as substrate by the method of Fishman, Springer & Brunetti (1948). This usually possessed an activity of about 700,000 Fishman units/g. The sulphatase activities of these preparations were not determined.

(c) Enzymic hydrolysis of urine in analytical work

The urine sample was acidified to pH 4.7 with glacial acetic acid and kept buffered to this pH by the addition of one tenth of its volume of M-acetate buffer. The enzyme dissolved in a small volume of water was then added, mixed well, and the mixture set aside at 37° for 24 hr.

(d) Micro-Girard separation

The urinary phenolic extract was dissolved in 2 ml. of ethanol and 2 ml. of glacial acetic acid. To this was added 20-50 mg. Girard's reagent T (trimethyl ammonium acetohydrazide chloride) and the reaction mixture was set aside at room temperature overnight. The following day the mixture was thoroughly chilled and 50 ml. of NaOH solution, which would neutralize 90% of the acid, were added. The aqueous phase was saturated with NaCl before extracting the non-ketonic fraction with 3 times 40 ml. of ether. The combined ether extract was washed with one twentieth of its volume of H_2O, which was added to the aqueous phase. The aqueous phase was acidified with 6 ml. of 10 N-HCl or 50% (v/v) H_2SO_4. After 1 hr. at room temperature the ketonic fraction was recovered by ether extraction (3 x 40 ml.). The combined ether extract was washed 3 times with one tenth of its volume of 8.5% $NaHCO_3$ and 3 times with one tenth of its volume of water, dried over Na_2SO_4 and evaporated to dryness.

(e) Partition chromatography

This was based on the method described by Bauld (1955). The two phases were equilibrated overnight. A 5 ml. portion of the stationary phase was thoroughly mixed into 5 g. of Celite. Sufficient mobile phase was then added to prepare a slurry which was packed into a 'micro' column, 1 cm. in diameter, to a height of 10 cm. 'Channelling' due to uneven packing was prevented by rotating the column during packing. The mobile phase should percolate through at a rate of 12-15 ml./hr.

The residues to be analysed were dissolved in mobile phase and a known aliquot pipetted on to the column. When the complete residue was to be analysed, quantitative transfer was effected by dissolving it in 1 ml. of mobile phase and pipetting the solution on to the column; this procedure being repeated twice after each transfer had percolated into the column. Collection of the eluate in 2 ml. portions was begun after the first 2 ml. had percolated through.

(f) The modified Kober reaction

The Kober reaction as modified by Brown (1952) and Bauld (1954) was used. The reagent employed, which was used by Bauld for the estimation of oestriol, was a 2% (w/v) hydroquinone solution in 76% H_2SO_4 containing 40 mg. of quinone and 20 mg. of $NaNO_3$. All determinations of Kober chromogens were expressed as oestriol equivalents.

3. The development of a system suitable for the identification of 16-oxooestradiol-17β in menstrual cycle urines.

The solvent system 70% (v/v) methanol in water/25% (v/v) ethylene dichloride in benzene used in the original detection of 16-oxo-oestradiol-17β in the ether-soluble phenolic-ketonic fraction from pregnancy urine did not give a clear-cut separation of oestrone from 16-oxo-oestradiol-17β. It was evident that another system which could overcome this deficiency should be made available before estimation of ten microgram quantities of KC-5 could be reliably carried out.

By progesssively making less 'polar' the mobile phase of the system mentioned above, first by reducing the concentration of ethylene dichloride and then replacing it with hexane, several suitable systems were arrived at empirically. It was found that benzene-hexane mixtures, viz., 90:10; 80:20; 75:25, all give separation of the two oestrogens. The system 70% methanol in water/benzene-hexane (4:1, by vol.) was finally adopted for quantitative work. This gives a zero elution zone of 6-8 ml. between/

between oestrone and 16-oxooestradiol-17β, the latter being eluted in the 16-32 ml. fraction.

Oestradiol-17β, when chromatographed in this system, came between oestrone and 16-oxo-oestradiol-17β with slight overlapping with each of them. 16-*Epi*oestriol and 17-*epi*oestriol were only eluted from about 40 ml. onwards.

Though no dioxo phenolic steroids like 16-oxooestrone have been examined in this system, it might be expected that most of them would appear in the fraction between oestrone and 16-oxooestradiol-17β.

Recoveries of both oestrone and 16-oxooestra-diol-17β from the columns were high, being 95-100% for oestrone and seldom less than 90% for the latter. In these and subsequent recovery experiments, unless otherwise stated, 50 μg. quantities of 16-oxooestradiol-17β were used.

4. Destruction of 16-oxooestradiol by ether peroxides.

Preliminary recovery experiments with the complete method were carried out with 16-oxo-oestradiol-17β added to aqueous acid solutions. Very/

Very poor recoveries were obtained. It was evident that serious losses occurred at one or several of the steps. It was therefore decided to investigate each of these in detail.

The extraction of oestrogens added to a solution containing 15% (v/v) concentrated HCl with ether has been shown to be quantitative. Thus it was most surprising to obtain recoveries of 4 and 19%, when 16-oxooestradiol-17β was used. The ether used in the extractions was at once suspected to be the cause of the serious losses.

Accordingly, 50 μg. quantities of 16-oxo-oestradiol-17β were each dissolved in 450 ml. of the same ether, together with some recovered from the previous distillations. These solutions were evaporated to dryness. The residues were then dissolved in alcohol and aliquots removed for the Kober reaction. Recoveries ranged from 49-62%.

Similar experiments, using recovered ether which had not been purified, were carried out with losses ranging from 30 to 80%. This seemed to indicate that the destruction was dependent on/

on the state of purity of the ether used in the experiments.

Ether freshly purified by shaking with alkaline silver oxide had very little destructive effect on 16-oxooestradiol-17β. Thus, in a comparative study of the relative stabilities of oestrone, oestradiol-17β, 16-oxooestradiol-17β and oestriol in ether purified in this manner, recoveries were practically quantitative.

Similarly, 16-oxooestradiol-17β was not destroyed to any significant extent in ether, which had its peroxides removed by previous treatment with ferrous sulphate solution. For example, recoveries of 16-oxooestradiol from ether before and after purification were 58% and 90% respectively. Even with the unpurified ether both 16-*epi*oestriol and oestriol were not destroyed.

These observations clearly suggested that the destruction was due to ether peroxides and that it involved a deep-seated modification of the 16-oxooestradiol molecule, with a consequent loss in Kober chromogenicity. The finding that 16-*epi*oestriol and oestriol were not affected under these conditions excluded the possibility that/

that ether residues might have inhibited the full development of the Kober colours (cf. Bauld, 1954), thus giving a false impression that destruction had actually taken place.

It is well known that ether undergoes a complex oxidation on standing, especially in the presence of light. Hence, in an attempt to confirm the conclusions mentioned above, an old batch of ether was refluxed gently for 4 hours under good illumination. Solutions of 16-oxo-oestradiol-17β in this 'treated' ether were further refluxed for half an hour before evaporation to dryness. Three such experiments gave recoveries of 32.6, 55.7 and 70% respectively. These variations were not surprising in view of the difficulties involved in controlling the experimental conditions. The results were not inconsistent with the idea that peroxides were responsible for the destruction of 16-oxooestradiol-17β.

An attempt was made to correlate the peroxide content of impure ether with the degree of destruction of 16-oxooestradiol-17β. Thus, by/

by treating equal quantities of 16-oxooestradiol-17β with two different batches of impure ether under as similar experimental conditions as possible, it might be expected that the ratio of the losses encountered would be equal to that of the peroxide contents, if a direct correlation exists. The peroxide content of each was determined by measuring the absorption of the ferric thiocyanate complex at 500 mμ which was formed when 10 ml. of the impure ether were shaken up with 2 ml. of a ferrous thiocyanate mixture (the latter in excess). Solutions of 16-oxooestradiol-17β in these ethers were simultaneously refluxed over the same water bath for 30 minutes, and finally distilled to dryness. The residues were assayed for Kober chromogens in the usual manner. The following table summarizes these results.

Table 1,II. /

Table 1.II.

Correlation of the peroxide content of impure ether with the degree of destruction of 16-oxo-oestradiol-17β.

Expt.No.	Ratio of O.D. at 500 mμ	Ratio of losses in %
1	2.80	2.87
2	1.15	1.25
3	1.35	1.18
4[x]	2	1.4

x Expt. 4 had no prior refluxing

These results indicate conclusively that the destruction of 16-oxooestradiol-17β is oxidative in nature.

The nature of the oxidation product of 16-oxo-oestradiol-17β.

That the product did not give a Kober colour was evident since both it and the unaffected 16-oxooestradiol-17β were together present in the residue after removal of the ether. In view of the finding of Marlow (1948) that 16-oxooestrone does not give a Kober colour it was considered likely that the oxidation product/

product might be 16-oxooestrone. If this were so, the possibility would therefore arise that 16-oxooestrone might be an artifact formed from 16-oxooestradiol-17β when ether containing peroxide is used in working up urinary oestrogen extracts. It may be recalled that Serchi (1953) has reported the isolation of 16-oxooestrone from menstrual cycle urine. More recently, Slaunwhite & Sandberg (1956) have reported the detection of 16-oxooestrone in the urine of a human subject treated with $[16-^{14}C]$ - oestrone. An attempt was therefore made to identify the peroxide oxidation product of 16-oxooestradiol-17β.

Huffman (1942) has described a sensitive colour reaction given by the dioxime of 16-oxo-oestrone with alcoholic copper acetate solution. The yellowish-green colour could be extracted into chloroform. Thus, the presence of 16-oxo-oestrone would be indicated if the residue after treatment with an aqueous alcoholic solution of hydroxylamine hydrochloride and sodium acetate gave on further addition of alcoholic cupric acetate/

acetate the characteristic yellowish-green colour. This was observed to be the case. 16-Oxooestradiol-17β did not produce any colour under these conditions. Likewise, a mixture of these reagents gave rise to a very pale yellow solution. The colour passed into chloroform when the diluted alcohol was extracted.

Thus it appeared that 16-oxooestrone was indeed formed during the oxidation of 16-oxo-oestradiol-17β. However, when a paper chromatographic technique was made available later (Marrian & Holdsworth, unpublished work; see Section IV), analysis of ether-treated residues from 16-oxooestradiol-17β showed no 16-oxooestrone but products more 'polar' than oestriol and hence these were presumably acidic. It was also shown that 16-oxooestrone is not stable towards impure ether.

It may be concluded, however, that ether peroxides bring about a rupture of ring D of 16-oxooestradiol-17β. Whether or not 16-oxooestrone is an intermediate in this oxidative degradation remains in doubt.

Means of eliminating losses of 16-oxooestradiol-17β through oxidation by ether peroxides.

As was shown above, destruction of 16-oxo-oestradiol-17β could be reduced to a very low level, provided peroxide-free ether is used. It has since been the writer's practice to test every batch of ether, even though it has been previously purified, with a mixture of ferrous sulphate and ammonium thiocyanate, before it is used for analytical work. Any ether that gives a red colour on shaking up with the ferrous thiocyanate mixture is rejected.

Since traces of water could catalyse the formation of ether peroxides, it is considered safer to dry all ether solutions with Na_2SO_4 before evaporation. This is particularly so when a few micrograms of KC-5 are to be determined.

With these simple precautions the recovery experiments in which 16-oxooestradiol-17β was added to and subsequently recovered from an acid solution, was repeated and the yields were satisfactory.

In view of the risk involved in evaporating large volumes of ether, it was decided to carry out the phenolic separation first instead of the Girard separation.

5. Removal of the 'acidic' fraction from the ether extract.

Brown (1955) has shown that the partition coefficient of oestriol between ether and concentrated carbonate buffer of pH 10.5 to be practically the same as that between ether and saturated $NaHCO_3$. The use of a concentrated carbonate buffer to remove the acidic fraction is superior to that of $NaHCO_3$ in that it removes more impurities. It was shown by Bauld (1952) that if the ether extract, after being washed with the concentrated carbonate buffer, is shaken with 2N-NaOH, and subsequently neutralized to about pH 10 with $NaHCO_3$ and reshaken again, more pigments pass into the aqueous phase. This simple procedure purifies the ether extract considerably more than a second wash with concentrated carbonate buffer, and it has been incorporated into both methods.

The simple bicarbonate wash used in the preceding recovery experiments resulted in nearly 100% recovery of 16-oxooestradiol-17β. However, with the concentrated carbonate buffer washing, the recoveries of 16-oxooestradiol-17β after/

after removal of the ether were consistently about 20% lower (4 experiments). It was evident that the latter purification step could not be used for 16-oxooestradiol-17β.

Though the reason for such losses was not obvious at that moment, it certainly could not be due to the unfavourable partition of 16-oxo-oestradiol-17β between ether and the concentrated carbonate buffer, as this procedure results in the complete retention of oestrone, oestradiol-17β and oestriol in the ether phase. The cause of these losses became apparent when it was found later that 16-oxooestradiol-17β is readily destroyed in strong alkaline solution.

6. Separation of the phenols from the neutrals

The next step to investigate was the phenolic separation. This could be carried out either (a) by extracting the ether extract (500 ml.) with 3 times 100 ml. of N-NaOH, or (b) by extracting the phenols in benzene solution (50 ml.) with 3 times 25 ml. of N-NaOH. The combined alkaline extracts from each of these two procedures were neutralized to pH 9.0 with/

with CO_2, and the phenols recovered by re-extraction with ether.

Comparison of these two methods led to the findings summarized in the following table.

Table 2,II

Recoveries of 16-oxooestradiol-17β from ether or benzene solution by alkaline extraction

	Recovery (%)		
From ether	80.8	80.2	72.3
From benzene	47.7	43.0	-

The much lower recoveries with method (b) were rather unexpected. That extraction of oestrogen from the organic phase was quantitative was demonstrated by the failure to detect any Kober chromogen in the ether or the benzene phase. It was noted that the alkaline extracts from method (b) were set aside at room temperature for a much longer time than those from (a) before neutralization with CO_2. These findings pointed to the possible conversion of 16-oxo-oestradiol-17β on prolonged standing in alkaline solution/

solution to non-Kober chromogenic substances.

Accordingly, the following study on the behaviour of 16-oxooestradiol-17β in alkaline solutions was carried out.

7. Destruction of 16-oxooestradiol-17β (and KC-5) in alkaline solutions.

In N-NaOH solutions. Solutions of 16-oxo-oestradiol-17β (50 μg.) in 300 ml. N-NaOH were allowed to stand at room temperature for various periods of time. They were then acidified with 5N-H_2SO_4 (slight excess) and extracted four times with 100 ml. of ether. After washing the ether extracts with bicarbonate solution, they were evaporated to dryness. Aliquots of the residues in alcoholic solution were taken for colorimetric assay. The following are the results obtained.

Table 3, II.

Recoveries of 16-oxooestradiol-17β from N-NaOH after various time intervals at room temperature.

Time in hr.	0	1	2
Recovery (%)	93.4	64.2	48.2

Table/

Table 4,II shows the recoveries from experiments carried out in a room at 0°C.

Table 4,II.

Recoveries of 16-oxooestradiol-17β from N-NaOH at various time intervals at 0°C.

Time in hr.	0^x	$\frac{1}{2}$	1	$1\frac{1}{2}$
Recovery(%)	84.2	92.7	89.1	83.7

x room temperature

Similarly, 16-oxooestradiol-17β was also readily destroyed in small volumes of N-NaOH (75 ml.) at room temperature. Thus, the following recoveries were obtained: 97.4% (0 hr.); 81.7% ($\frac{3}{4}$ hr.); 0.22% (3 hr.).

These experiments showed conclusively that 16-oxooestradiol-17β was destroyed by alkali and that this destruction could be minimized by carrying out the extraction with cold alkali as rapidly as possible. Another preventive measure was to acidify each extract immediately with cold 5 N-H_2SO_4. With these precautionary modifications the phenolic separation was reinvestigated. Method (a) gave recovery of about 93% while (b) gave 92%.

It/

It may be mentioned that 16-oxooestradiol-17β was not destroyed in 1.5 N-Na_2CO_3 solution at room temperature over a period of 2 hours.

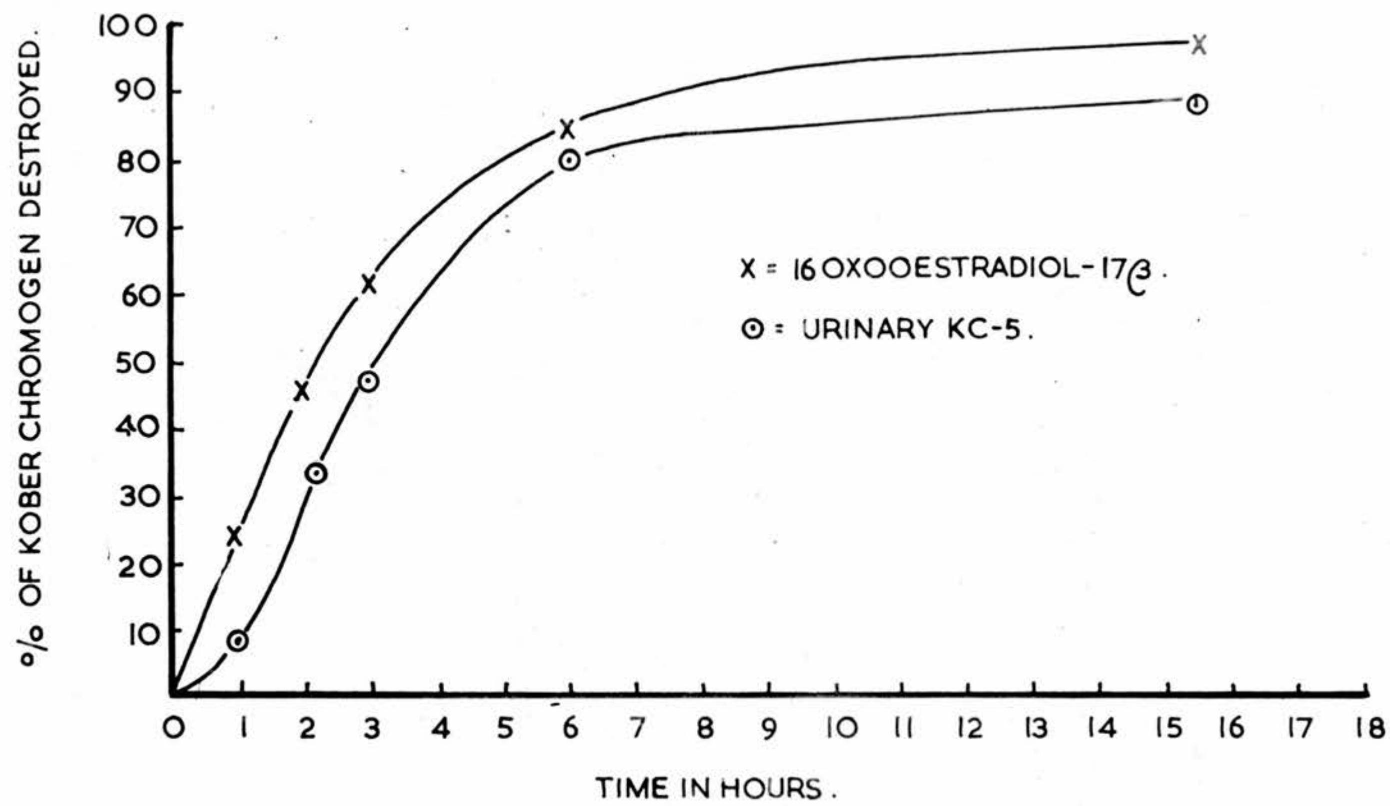
Comparison of the stabilities of urinary KC-5 and 16-oxooestradiol-17β.

Meanwhile ,characterization studies on KC-5 isolated from pregnancy urine seemed to indicate that the KC-5 might not be 16-oxooestradiol-17β. If this were so, it might well be that KC-5 would be stable to alkali after all. Moreover, the presence of other urinary compounds might prevent significant destruction of KC-5 during the alkaline fractionation. These two considerations alone made a comparative study of the behaviour of urinary KC-5 and 16-oxooestradiol-17β in alkaline solution desirable.

The KC-5 was obtained by partition chromatography of an ether-soluble phenolic-ketonic fraction from enzymically hydrolysed pregnancy urine in the system 70% methanol in water/benzene-hexane (4:1, by vol.). The Kober chromogen content of the KC-5 fraction was estimated and aliquots of the alcoholic solution, each/

FIG. 1, II

DESTRUCTION OF 16 OXOOESTRADIOL-17β & KC-5 IN N-NaOH SOLUTION.



each containing 50 μg. of Kober chromogen, were withdrawn. The residues were dissolved in N-NaOH and set aside at room temperature for various time intervals, after which the phenols were recovered in the usual manner. A parallel experiment with 16-oxooestradiol-17β was carried out. Fig. 1,II summarizes the results obtained.

It will be noted that destruction of KC-5 also occurred, but appeared to proceed at a slower rate during the early stage. The sigmoid part of the curve of urinary KC-5 also indicates that KC-5 was different from 16-oxooestradiol-17β, but in the latter stage of the reaction its behaviour was indistinguishable from that of 16-oxooestradiol-17β. This interesting fact was accounted for when urinary KC-5 was later found to contain mainly 16α-hydroxyoestrone and some 16-oxooestradiol-17β. 16α-Hydroxyoestrone itself undergoes rearrangement readily to 16-oxooestradiol-17β in alkaline solution (Section III). The 16-oxo-oestradiol-17β thus formed was of course destroyed by the alkali.

The/

The nature of the alkaline degradation product of 16-oxooestradiol-17β.

The destruction of 16-oxooestradiol-17β in alkaline solution was shown to be reduced somewhat (by about 10%) if the air was displaced with N_2. Appreciable destruction still occurred in view of the difficulty of removing all the dissolved oxygen. However, the evidence was sufficiently strong that oxygen played an active role in the alkaline degradation of 16-oxo-oestradiol-17β. It was therefore considered likely that 16-oxooestrone might be the first product formed. Attempts to detect 16-oxooestrone by means of the colour reaction described in (4), in the phenolic residues failed. On the other hand, the bicarbonate washings of the ether extract on acidification and re-extraction with ether afforded material, which gave a greenish-blue colour with a 1:1 mixture of 1% solutions of $K_3Fe(CN)_6$ and $FeCl_3$ (spot test for phenolic groups, cf. Barton, Evans & Gardiner, 1952). Thus, it appeared that the main product was a phenolic acid, arising from the ring D rupture of 16-oxooestradiol-17β.

Since/

Since 16-oxooestradiol-17β is stable in carbonate solution, it seems probable that the hydroxyl ion concentration has a very important role to play in the destruction of 16-oxooestradiol-17β. It may well be that the enol form of 16-oxooestradiol-17β, which presumably exists predominantly in strongly alkaline solutions, undergoes oxidation to 16-oxo-oestrone (cf. the oxidation of ascorbic acid to dehydroascorbic acid), which then undergoes alkaline cleavage to yield a dicarboxylic acid, namely, marrianolic acid. The failure to detect 16-oxooestrone may be ascribed to the lability of the dioxo grouping in alkaline solution. The finding of Touchstone, Elliot, Thayer & Doisy (1955) that in alkaline solutions and in the presence of O_2 both 16-oxooestradiol-17β and 16-oxooestrone give rise to the same product, marrianolic acid, in yields of about 70%, supports these conclusions.

8./

8. The choice of a method for the phenolic separation.

Both methods (a) and (b) have been shown to give good recoveries of 16-oxooestradiol-17β, provided the time for the extraction with alkali and subsequent neutralization of the extracts is kept as short as feasible.

In view of the deleterious action of ether peroxides, evaporation of large volumes of ether was considered undesirable; hence, method (a) was adopted. On the other hand, to remove the oestrogen from a large volume of ether also requires a proportionate increase in the volume of the alkali used in the extraction. Whether the losses arising from ether peroxides are more serious than those arising during the alkaline extractions have not been thoroughly investigated. It was hoped that these were small in any case. However, it was subsequently found that complete recovery of 16-oxooestradiol from ether could be effected by extracting three times with one-tenth of its volume of N-NaOH (instead of one-fifth). Using 0.1 N-NaOH for the extraction of 16-oxooestradiol-17β from either/

either benzene or ether did not yield quantitative recoveries.

9. The Girard separation.

Watson & Marrian (1955) have found that 16-oxooestradiol-17β is not stable under the conditions of the hot Girard reaction. Whether these poor recoveries were due to the formation of an azine, as was observed in the case of oestrone with Girard P or T reagents (Cohen, Bates & Lieberman, 1952) is not known.

However, by carrying out the Girard reaction at room temperature overnight high recoveries could be readily obtained. Their procedure is the same as described in General Methods, except that pre-saturation of the aqueous phase with NaCl was not included. Recoveries obtained in several experiments are given in Table 5,II.

Table 5,II /

Table 5.II

Recoveries of 16-oxooestradiol-17β in the ketonic fraction after the Girard separation.

Expt.	Amount of Girard T reagent added (mg.)	Recovery (%)
	10	72.8
A	20	51.4
	40	70.2
B	20	78.3
	20	86.7

It was evident that these losses were not due to incompleteness of the reaction but were mainly mechanical ones. It was pointed out by Professor Marrian that these variable losses might be attributed to the fine emulsions that were formed during the ether extraction of the non-ketonic fraction. Saturation of the aqueous phase with NaCl prior to extraction should prevent some of the water-soluble hydrazones from being carried into the non-ketonic fraction. Accordingly, saturation with NaCl was carried out and the recoveries obtained in consequence were very high indeed (nearly 100%).

Zaffaroni, Burton & Keutmann (1949) have pointed out that incubation at 40° of the Girard reaction mixture gave more satisfactory results than/

than the hot Girard reaction. Some Girard reactions were hence carried out at 37°, and it was found that the reaction was essentially complete with little loss of 16-oxooestradiol-17β in 3-4 hours. However, owing to the time factor it is inconvenient to incorporate this alternative procedure into the complete method.

10. A method for the quantitative determination of 16-oxooestradiol-17β.

Briefly, the method arrived at as a result of these step-by-step studies is as follows:-

A urine specimen (500 ml.) was hydrolysed either by acid or enzymically. The urine was then extracted once with 150 ml. and three times with 125 ml. of peroxide-free ether. The combined ether extract was washed twice with 50 ml. of 8.5% (w/v) $NaHCO_3$ and extracted three times with 50 ml. chilled N-NaOH. Each of the alkaline extracts was immediately neutralized with 5 N-H_2SO_4. When cool, the acidified solution was then extracted four times with 50 ml. peroxide-free ether and the combined ether extract/

extract was washed twice with 20 ml. $NaHCO_3$ and three times with 20 ml. water, dried and evaporated to dryness.

The Girard separation was carried out as described in General Methods.

The phenolic-ketonic residue thus obtained was chromatographed on a micro column using the solvent system 70% methanol in water/ benzene-hexane (4:1, by vol.). Successive 2 ml. fractions were collected in Kober tubes and the residues obtained after removal of solvents were assayed colorimetrically. The KC-5 fraction might, if desired, be collected in one fraction (16-32 ml.).

Several full recovery experiments with 50 μg. 16-oxooestradiol-17β added to aqueous solution were carried out with an overall recovery of about 90% (average of 5).

11. To investigate further the stability of 16-oxooestradiol-17β in hot acid urines.

The conditions of acid hydrolysis have been well standardized as a result of the efforts of numerous workers in oestrogen methodology. It was therefore felt that further investigations on/

on the stability of 16-oxooestradiol-17β in acid hydrolysed urines should be made before finally abandoning this useful tool for enzymic hydrolysis.

Moreover, with the demonstration that with proper precautions no serious loss of 16-oxo-oestradiol-17β could occur in all the stages following acid hydrolysis, the time had arrived for a re-investigation of this problem. For instance, it would be possible to estimate the extent of destruction actually occurring during hydrolysis by adding 16-oxooestradiol-17β to the urine before and after acid hydrolysis. Consequently, if the losses were not too serious, it was hoped to investigate whether they could be prevented or minimized in some way. This aim, if fulfilled, would permit the retention of a well-tried and easily standardized procedure.

Using the method as described in (10), two sets of recovery experiments were carried out. In (T) the 16-oxooestradiol-17β was added to a 500 ml. portion of a diluted 24 hour male urine specimen before acid hydrolysis, while in (C) it/

it was added to the cooled acid-hydrolysed urine. A urine blank was also carried out. The results are given in Table 6,II.

Table 6,II.

Recoveries of 16-oxooestradiol-17β added to urine before hydrolysis (T) and after hydrolysis (C).

Expt. No.	Recovery (%)	
	(T)	(C)
1	33.0	18.0
2	14.2	32.0

The interesting fact thus emerged that 16-oxooestradiol-17β is just as readily destroyed in hydrolysed urine as in urine during hydrolysis. To confirm this observation, the following two sets of experiments were carried out. In (T) the oestrogen was added to the cooled hydrolysed urine, while in (C) it was added to unhydrolysed urine.

Table 7,II /

Table 7.II

Recoveries of 16-oxooestradiol-17β added to hydrolysed (T) and unhydrolysed (C) urine.

Expt. No.	Recovery (%) (T)	(C)
1	27.7	90
2	32.4	71 x

x Unhydrolysed urine + 75 ml. conc. HCl; serious emulsions formed on extraction with ether.

It seemed certain that 16-oxooestradiol-17β was destroyed by some agent(s) which was formed from some urinary constituents during acid hydrolysis, since unboiled urines gave fairly satisfactory recoveries. The results seemed to indicate that the destruction must be fairly rapid in acid-hydrolysed urine, since extraction with ether was usually carried out within 5 minutes after the addition of 16-oxo-oestradiol-17β. On the other hand, it is also possible that destruction might have proceeded through all the subsequent stages so long as the destructive/

destructive agent(s) and 16-oxooestradiol-17β were not separated from one another. However, quantitative recoveries of 16-oxooestradiol-17β added to a phenolic residue as well as to a phenolic-ketonic residue seemed to indicate that the destruction occurred mainly in the urine and probably also in the ether extract.

The destruction of 16-oxooestradiol-17β in acid solution containing urea.

The preceding experiments pointed to a possible means of overcoming or minimizing these serious losses occurring during acid hydrolysis. For, if the progenitor of the destructive agent could be removed from the urine before acid hydrolysis, subsequent boiling of the acid urine with added 16-oxooestradiol-17β would presumably result in little destruction. For some obscure reason, our attention was focussed on urea - one of the major constituents of urine - and it was decided to study the stability of 16-oxooestradiol-17β in acid solution containing urea.

In these experiments 7 g. of urea (roughly equal/

equal to one fourth of the daily urinary excretion) were added to an aqueous solution (500 ml.) of 16-oxooestradiol-17β (50 μg.). The solution was hydrolysed with 15% (v/v) conc. HCl in the usual manner. The substance was recovered by extraction with ether and was assayed colorimetrically. Nearly one half of the 16-oxooestradiol-17β was lost (2 experiments). In view of the high background colours in the Kober reaction this experiment was repeated and the product was assayed after purification by partition chromatography. The losses were about 5% higher. Adding 16-oxooestradiol-17β to the cooled acid-hydrolysed urea solution yielded a recovery of 88%.

Urinary KC-5 was also destroyed to nearly the same extent under the same conditions.

These findings are interesting in that theoretically the urea could be removed from the urine either by treatment with nitrous acid or sodium hypobromite, or by the action of urease. However, practical difficulties were anticipated and it was decided to make a different approach towards the solution of this problem.

To/

To investigate whether destruction of 16-oxo-oestradiol-17β occurs during the acid hydrolysis of urinary conjugates.

It was hoped that by making a urinary conjugate extract most of the potentially destructive substances would remain in the urine. Acid hydrolysis of these urinary conjugates would prove less destructive for 16-oxo-oestradiol-17β. Some preliminary experiments were therefore carried out.

An extract of urinary conjugates was made from 500 ml. of male urine according to the procedure of Edwards, Kellie & Wade (1953).

The semi-crystalline brown residue obtained after removal of the solvents was dissolved in 500 ml. of water. After adding 50 μg. of 16-oxo-oestradiol-17β, the mixture was hydrolysed by acid. The 16-oxooestradiol-17β was recovered following the method described in (10).

As a comparison, another acid hydrolysis was carried out on 500 ml. of the same batch of urine containing 50 μg. of 16-oxooestradiol-17β.

The results are given in the following table.

Table 8,II /

Table 8.II.

Recoveries of 16-oxooestradiol-17β added to urine and urinary conjugates before hydrolysis.

Expt.	Recovery (%) Urine	Conjugates
1	5.5	17.6
2	17.2	23.0

Though there were indications that 16-oxo-oestradiol-17β was destroyed to a lesser extent during hydrolysis of the urinary conjugates than during hydrolysis of the whole urine, it was evident that the recoveries were still too poor to justify any optimism. For one thing the recoveries were in fact lower than some of the previous experiments on whole urines. Moreover, the preparation of the urinary conjugate extract was time-consuming and by no means a quantitative procedure. In the light of such considerations it was felt that efforts would be more profitably spent in studying the optimum conditions for enzymic hydrolysis.

12. /

12. To determine the optimum conditions for enzymic hydrolysis of oestrogen conjugates.

The use of β-glucuronidase preparations from Patella vulgata for hydrolysis of glucuronides has become quite widespread since Dodgson, Lewis & Spencer (1953) demonstrated the richness of sulphatases and β-glucuronidases in the visceral hump. However, little has been done to determine the optimum conditions for enzymic hydrolysis of oestrogen conjugates in urine.

In large-scale isolation work enzymic hydrolysis is allowed to proceed for 48 hours at 37°. However, for convenience sake it was decided at the start to limit the period of hydrolysis to 24 hours in quantitative work. In view of the sensitivity of the enzyme to inhibition (non-competitive and competitive), the amount of enzyme to be used would have to be determined to ensure complete hydrolysis of the oestrogen conjugates within this curtailed period of time. For the following study a water-soluble enzyme preparation obtained as described in Section II,2 from P. vulgata was used/

used. Late pregnancy urine was used as the source of oestrogen conjugates.

In a first series of experiments to determine the optimum enzyme concentration, serious difficulties were encountered with emulsion formation in the extraction procedure. Addition of NaCl or $(NH_4)_2SO_4$ to the enzymically hydrolysed urine prior to extraction was of no avail. Neither did acidification to pH 1 with HCl effect the disappearance of these emulsions. It was subsequently found that extraction of the urine with twice its volume of ether produced little emulsion. Extraction with an equal volume of ether would also do, but was less satisfactory. Later, we were informed that Bradosol, a commercially available product, prevents the formation of emulsion, and by adding this to the enzymically hydrolysed urine, extraction with an equal volume of ether could be satisfactorily carried out.

(a) Effect of enzyme concentration.

To 100 ml. portions of pregnancy urine buffered to pH 4.7 with 10 ml. M-acetate buffer were added aliquots of the enzymic solution (100,000 units/ml.). The mixtures were then incubated/

incubated at 37° for 24 hours, then boiled for 1 min. and finally cooled under the tap. Each was extracted twice with 250 ml. of ether. The combined ether extract was washed twice with 40 ml. 8.5% $NaHCO_3$ and then extracted twice with 50 ml. N-NaOH (cold), each extract being acidified immediately with cold 5 N-H_2SO_4. After cooling the acidified solution was extracted three times with 60 ml. of ether. The combined ether extract was washed twice with 15 ml. 8.5% $NaHCO_3$ and three times with 15 ml. water, dried and evaporated to dryness.

The phenolic residues were separated into ketonic and non-ketonic fractions in the usual manner.

Portions of the phenolic-ketonic residues were chromatographed on micro Celite column, using the system 70% methanol in water/benzene-hexane (4:1, by vol.). The fractions were assayed. The results are shown in Fig. 2,II.

A concentration of 200,000 Fishman units/ 100 ml. of urine seemed to effect optimum hydrolysis of KC-5. It was thus chosen for the following study on the effect of pH on enzymic hydrolysis/

FIG. 2, II.

EFFECT OF ENZYME CONCENTRATION ON THE HYDROLYSIS OF KC-5 CONJUGATES IN PREGNANCY URINE.

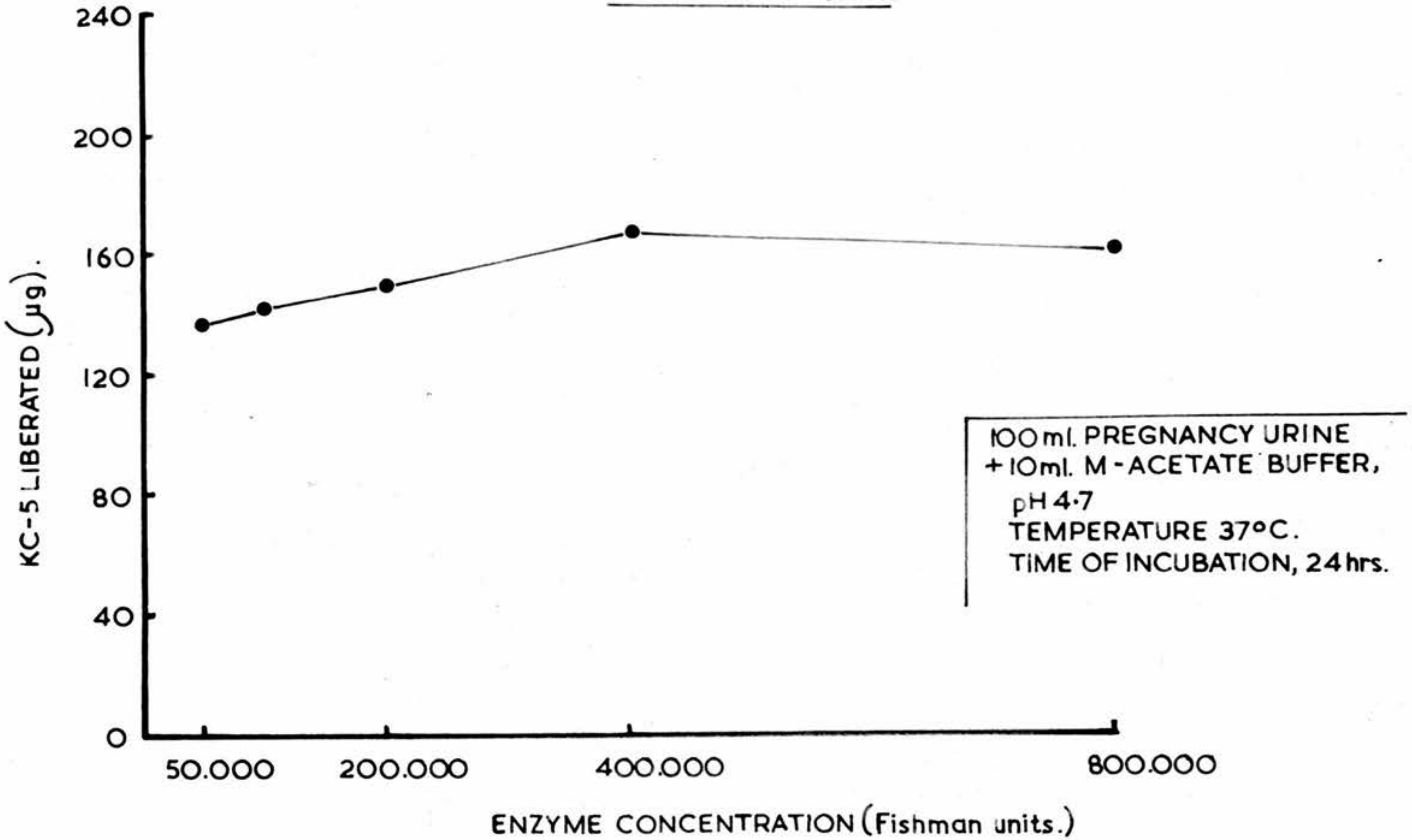


FIG. 3, II

EFFECT OF pH ON ENZYMIC HYDROLYSIS OF KC-5 CONJUGATES.

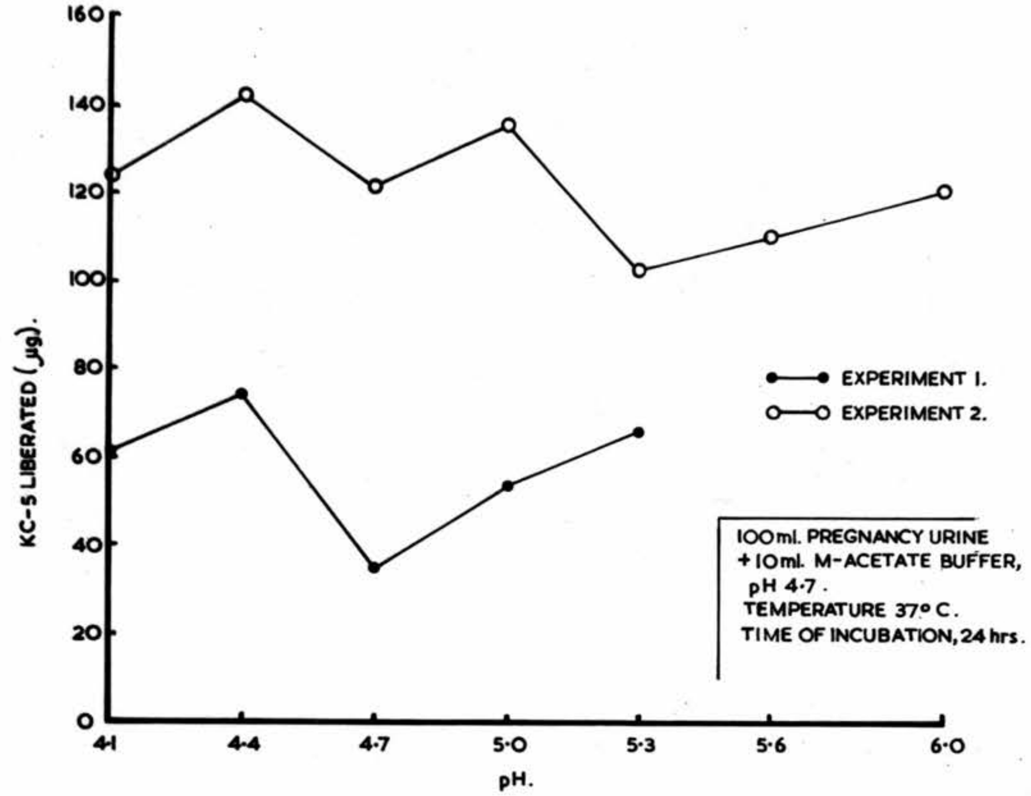
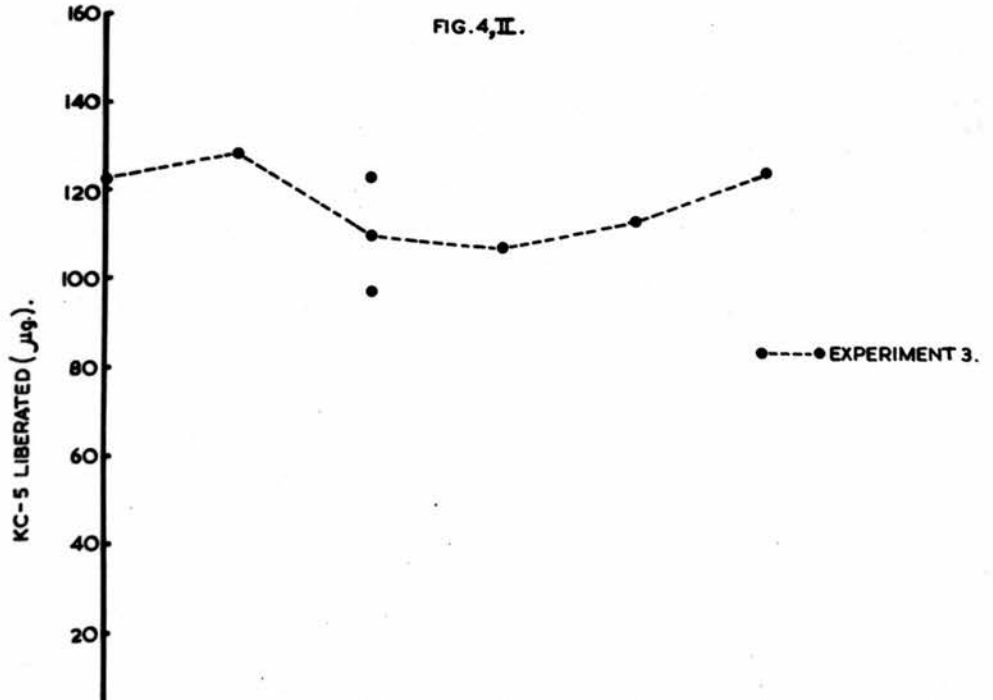


FIG. 4, II.



hydrolysis.

(b) Effect of pH.

Five 100 ml. portions of pregnancy urine, buffered to pH 4.1, 4.4, 4.7, 5.0 and 5.3 respectively were each incubated with 200,000 Fishman units of the enzyme for 24 hr. at 37°. The phenolic-ketonic fractions were worked up and assayed as described in (a).

In Fig. 3,II it will be noted that the yield of KC-5 at pH 4.7 was significantly lower than those at pH 4.4 and 5.0.

Accordingly two more sets of experiments were carried out with different pools of late pregnancy urine. The procedure was the same except that no boiling was carried out after hydrolysis. The results are summarized in Figs. 3,II (curve 2) and 4,II.

There is some indication that highest yields of KC-5 were obtained at pH 4.4. However, the failure to obtain reasonably good agreement in the results of the duplicate experiments at pH 4.7 (Expt. 3) cast some doubt on the results obtained in experiments 1 and 2. It was felt that/

that these fluctuations might be due to technical and manipulative losses and hence no pH minimum occurred at pH 4.7. It was thus decided to discontinue investigating this pH effect until a reliable and accurate method is available. In any case in urines with low oestrogen titres the use of a high enzyme concentration might offset the disadvantage of its having to work at a suboptimal pH and thus would result in complete hydrolysis of the oestrogen conjugates.

It might be mentioned that in the course of analysing such chromatographic fractions a new Kober chromogen (KC-6) more polar than KC-5 was detected.

13. Recovery experiments with urinary KC-5 added to enzymically hydrolysed male urine.

Some recovery experiments were carried out to determine whether high enzyme concentrations interfered with the recovery of added oestrogens. The procedure and the volumes of urine, etc. were exactly the same as described in 12(a) except that no boiling was carried out after hydrolysis/

hydrolysis. Urinary KC-5 containing the required amount of Kober chromogen calculated as oestriol was added to the enzymically hydrolysed urine. Urine controls (in duplicate) were set up. The experiments carried out in duplicate yielded results given in Table 9,II.

Table 9,II

Recoveries of urinary KC-5 added to enzymically hydrolysed urine.

Amount of Kober chromogen added (μg.)	Recovery (%)
50	(i) 82.5 (ii) 86
25	(i) 89 (ii) 72
12.5	(i) 75 (ii) 66

14. Formation of an oestrone-like compound by 16-oxooestradiol-17β in alcoholic solution on prolonged standing.

An interesting fact was discovered when a pure 16-oxooestradiol-17β standard, previously prepared in alcohol and stored at 0° for about a month, was chromatographed on Celite as described/

described in (3). An 'oestrone-like' Kober chromogen was detected. When this observation was first made, the ratio of the oestrone-like compound to 16-oxooestradiol-17β was 1:2.5.

The oestrone-like compound might be due to (a) internal cyclization of the ketolic structure with subsequent etherification, or (b) the formation of a hemiketal or ketal between 16-oxooestradiol-17β and alcohol - both processes probably being catalysed by traces of acid in the solution. If this were so, it might be expected that the oestrone-like compound would be converted back to 16-oxooestradiol-17β in aqueous or aqueous acid solution

Accordingly three weeks later the following experiment was carried out. A solution of 50 μg. of 16-oxooestradiol-17β, found to contain 33.3% of the oestrone-like compound on the same day, in 50 ml. of water was allowed to stand at room temperature for 30 minutes. The recovered product was then analysed by partition chromatography. The ratio of the oestrone-like compound to 16-oxooestradiol-17β was altered to 1:6.

Treatment/

Treatment with 0.5 N-H_2SO_4 for 2 hr. did not alter the originally observed ratio much. However, after 17 hr. at room temperature, the ratio was 1:19.

It thus appears that either of the processes (a) and (b) may occur in solution. In this connection the recent work of Wheeler (1957) is noteworthy. He found that cyclopentanone reacted readily with methanol in the presence of HCl to yield the hemiketal; ethanol was less effective while cyclohexanone reacted sluggishly with acid methanol.

The same phenomenon was recently discovered by my colleague, Dr Watson, in another standard solution of 16-oxooestradiol-17β.

15. Discussion

It is evident from the results and observations presented in this section that the difficulties associated with the quantitative determination of 16-oxooestradiol-17β, though numerous, are not insurmountable. The recovery experiments with unboiled acid urines yielded very satisfactory results at the 50 µg. level.
The/

The few experiments with urinary KC-5 added to enzymically hydrolysed urines were satisfactory too. Thus, it is expected that with improvements and/or certain modifications a method would be found which could be applied to the determination of KC-5 in menstrual cycle urines. Such a method would of course have to possess the necessary reliability characteristics in regard to accuracy, precision, sensitivity and specificity. However, it is the writer's belief that the present method could be applied to urines in which a reasonably high level of KC-5 excretion is expected, for example, in metabolic studies.

There is reason for some optimism that a method for the determination of a few μg. of KC-5 would be eventually evolved, for, as is now known, the main component in KC-5 is 16α-hydroxyoestrone with some 16-oxooestradiol-17β. Besides these, Professor Marrian and Dr Layne (work to be published) have recently shown that a third α-ketolic oestrogen, namely, 16β-hydroxyoestrone,is present in neutral-phenol fractions from/

from pregnancy urine in quantities somewhat less than that of 16-oxooestradiol-17β. It may be reasonable to assume that in urine of low oestrogen titres the same concentration relationship holds for the three ketols present. While 16α-hydroxyoestrone is only about 20% isomerized to 16-oxooestradiol-17β in alkaline solution, its 16β-epimer is entirely rearranged in 10 minutes. Thus, in a KC-5 extract there might be nearly equal amounts of 16α-hydroxyoestrone and 16-oxooestradiol-17β. Insofar as destruction of 16α-hydroxyoestrone in alkaline solution probably involves a prior rearrangement to 16-oxooestradiol-17β, this brief spell serves to spare it from oxidative destruction. Thus, in carrying out the recovery experiments with 16-oxooestradiol-17β, we were actually working under conditions in which recovery was less favourable. It might therefore be expected that recoveries with pure 16α-hydroxyoestrone should yield better results. Recent investigations with synthetic 16α-hydroxyoestrone carried out by Dr Watson have shown that/

that this is so.

It has been shown that the concentrated carbonate method for the separation of the acidic fraction from the ether extract gives rise to nearly 20% loss in 16-oxooestradiol-17β. It thus seems that the brief time during which the compound is exposed to strong alkali before being extracted into ether again, is long enough to cause this destruction. Hence, a very useful procedure for the purification of urinary phenolic fractions has to be dropped. Similarly, the final saponification step used by Bauld (1956) to remove most of the coloured contaminants, or the oxidation of urinary pigments with hydrogen peroxide in the Brown method, cannot be incorporated in the method for the determination of KC-5. It might therefore be expected that the final KC-5 fraction would still be grossly contaminated with substances which interfere with the Kober reaction. However, it appears that the sensitivity and the specificity of the method when applied to male urine are not seriously affected. This might not be so with other types of urine.

One/

One important modification that should be considered is the omission of the alkaline fractionation. This has been done with no obvious disadvantages in the isolation of 16β-hydroxyoestrone from pregnancy urine by Marrian & Layne and needs be considered seriously if determination of the three ketols is desired.

The destruction of 16-oxooestradiol-17β in acid solution in the presence of urea is of interest. Whether destruction involves ring A is not certain, for unfortunately similar studies with oestriol or oestrone have not been carried out. However, the condensation of α-ketols with amino compounds, albeit under anhydrous conditions, has been demonstrated by Julian, Meyer, Magnani & Cole (1945) and by Hurd & Buess (1956). Moreover Boscott (1955) has demonstrated the presence of hydantoins in acid-hydrolysed urines, the formation of which depends on the condensation of α-ketoacids with urea in the presence of acid. It may therefore be postulated that the losses occurring during acid hydrolysis of urine (and urinary conjugates) might partly arise from the condensation/

condensation of the α-ketolic grouping of 16-oxooestradiol-17β with nitrogen-containing compounds. The same might also occur under the conditions of the hot Girard reaction. Moreover, the finding that 16-oxooestradiol-17β was quantitatively recovered from unboiled acid urines but not from cooled hydrolysed urines is consistent with this hypothesis, since most of the amino compounds are excreted in conjugate forms and only undergo condensation reactions after the reactive groups are freed by hydrolysis. Though no direct evidence is forthcoming, oxidative destruction of 16-oxooestradiol-17β also probably occurs during acid hydrolysis.

SECTION III. PREPARATION OF 16-HYDROXYOESTRONES

A. Preparation of 16α-Hydroxyoestrone and some Related Studies

Introduction

Characterization studies on crystalline KC-5 isolated from pregnancy urine led Marrian, Watson & Panattoni (1957) to the conclusion that the principal component in KC-5 was 16α-hydroxyoestrone contaminated with some artifactually formed 16-oxooestradiol-17β. To establish this without doubt the isolation of pure 16α-hydroxyoestrone from KC-5 and its comparison with authentic material was essential.

Moreover, if the above postulate were correct, synthetic 16α-hydroxyoestrone should preferably be used in recovery experiments like those described in the previous section, for there exist significant differences in reactivity and stability between urinary KC-5 and 16-oxo-oestradiol-17β (cf. Marrian _et al._ 1957; Section II), even though they are analogous to each other in general behaviour. The use of 16α-hydroxy-oestrone in the recovery experiments would therefore/

therefore provide a truer picture of the reliability of the quantitative method to be developed.

This section of the thesis describes the preparation of 16α-hydroxyoestrone from its diacetate, which has been described by Leeds, Fukushima & Gallagher (1954). Certain studies carried out with the synthetic material in connection with the urinary KC-5 problem are also described. In addition, 17-*epi*oestriol has been obtained by the catalytic reduction of 16α-hydroxyoestrone.

Results

Preparation and properties of 16α-hydroxyoestrone diacetate

16α-Hydroxyoestrone diacetate, m.p. 172-174.5°, was prepared from oestrone by the method of Leeds, Fukushima & Gallagher (1954). These workers recorded m.p. 179-180° for their preparation. However, it would appear that their preparation and ours were different polymorphic forms of the same substance, since a sample of the preparation of/

of Leeds _et al._ (1954) kindly supplied to us by Dr Gallagher was found to melt at 168.5-172°, and this melting point was not depressed after admixture with our own preparation. Moreover, Dr Gallagher has informed us that he has also observed a lower melting modification of the substance. Incidentally, it may be mentioned that the m.p. of pure KC-5 acetate obtained from urine after acetylation was 172.5-174.5°. The identity of KC-5 acetate and 16α-hydroxyoestrone diacetate has already been conclusively established (Marrian, Loke, Watson & Panattoni, 1957).

Reduction of the diacetate by sodium borohydride, followed by alkaline hydrolysis, yielded two products, which were indistinguishable from oestriol and 16-_epi_oestriol respectively on partition chromatography in the system 70% methanol in water:ethylene dichloride. Based on the Kober colorimetric assay, the yield of the 16-_epi_-oestriol-like product was about 15-19%. The major product was isolated and identified as oestriol by a mixed melting point with authentic material/

material, and, in another experiment, was identified after acetylation by a mixed melting point with authentic oestriol triacetate.

As is evident from Table 1, IIIA(p. 75), the yield of the 16-<u>epi</u>oestriol-like product was not significantly altered either by repeated crystallizations of the starting material or by varying the experimental conditions. The former appeared to preclude the presence of either 16β-hydroxyoestrone diacetate or 16-oxooestradiol-17β diacetate in the starting material, both of which could be expected to yield mainly 16-<u>epi</u>oestriol on reduction by sodium borohydride followed by hydrolysis. The possibility that 16-<u>epi</u>oestriol was formed as a result of rearrangement of 16α-hydroxyoestrone diacetate to 16-oxooestradiol-17β diacetate during the reduction, seemed to have little support from the latter finding. Thus it would seem that the reduction of the 17-oxo group in 16α-hydroxyoestrone diacetate is less stereochemically specific for the formation of a 17β-hydroxyl group than it is in oestrone (cf. Biel, 1951) and that 17-<u>epi</u>oestriol (Prelog, Ruzicka & Wieland/

Wieland, 1945), which is not clearly distinguishable from 16-*epi*-oestriol in the solvent systems used, was the 16-*epi*oestriol-like product which was formed.

Preparation of 16α-hydroxyoestrone

Acid hydrolysis of the diacetate was carried out as described by Leeds *et al.* (1954) for the rearrangement and hydrolysis of 16α:17α-epoxy-androstane-3β:17β-diol diacetate to 17-oxo-androstane-3β:16α-diol. After recrystallization the product melted at 238-240.5°, with marked shrinkage at about 216°, and had $[\alpha]_D^{16}$ + 180° (ethanol). The acetate prepared from this material melted at 169-173° and had $[\alpha]_D^{20}$ + 149 (ethanol). It did not depress the melting point of 16α-hydroxyoestrone diacetate. It was evident, therefore, that the hydrolysis product of the diacetate was indeed 16α-hydroxyoestrone.

Treatment of 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate with methanolic sulphuric acid at room temperature also afforded 16α-hydroxyoestrone. This is a more convenient procedure, and was employed when only the free compound was required.

Rearrangement/

Rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β at or near its melting point

The melting point of 16α-hydroxyoestrone, like that of the crude KC-5 isolated by Marrian, Watson & Panattoni (1957), was not depressed after admixture with 16-oxooestradiol-17β (m.p. 239-241.5°). It was therefore considered likely that at or near its melting point 16α-hydroxyoestrone might undergo rearrangement to 16-oxooestradiol-17β. On re-determining the melting point of 16α-hydroxyoestrone with a more rapid rate of heating over the range of 205-230° it was found that the shrinkage previously observed at about 216° was in fact a fairly sharp melt followed immediately by re-solidification, which was complete at about 220°. This finding suggested that the lower melting point might be the true one for 16α-hydroxyoestrone and that the higher one might be that of 16-oxooestradiol-17β. This was shown to be true when 16-oxooestradiol-17β, which was identified by its physical properties and those of its diacetate, was isolated from the melt obtained by heating 16α-hydroxyoestrone at 220-230° *in vacuo*.

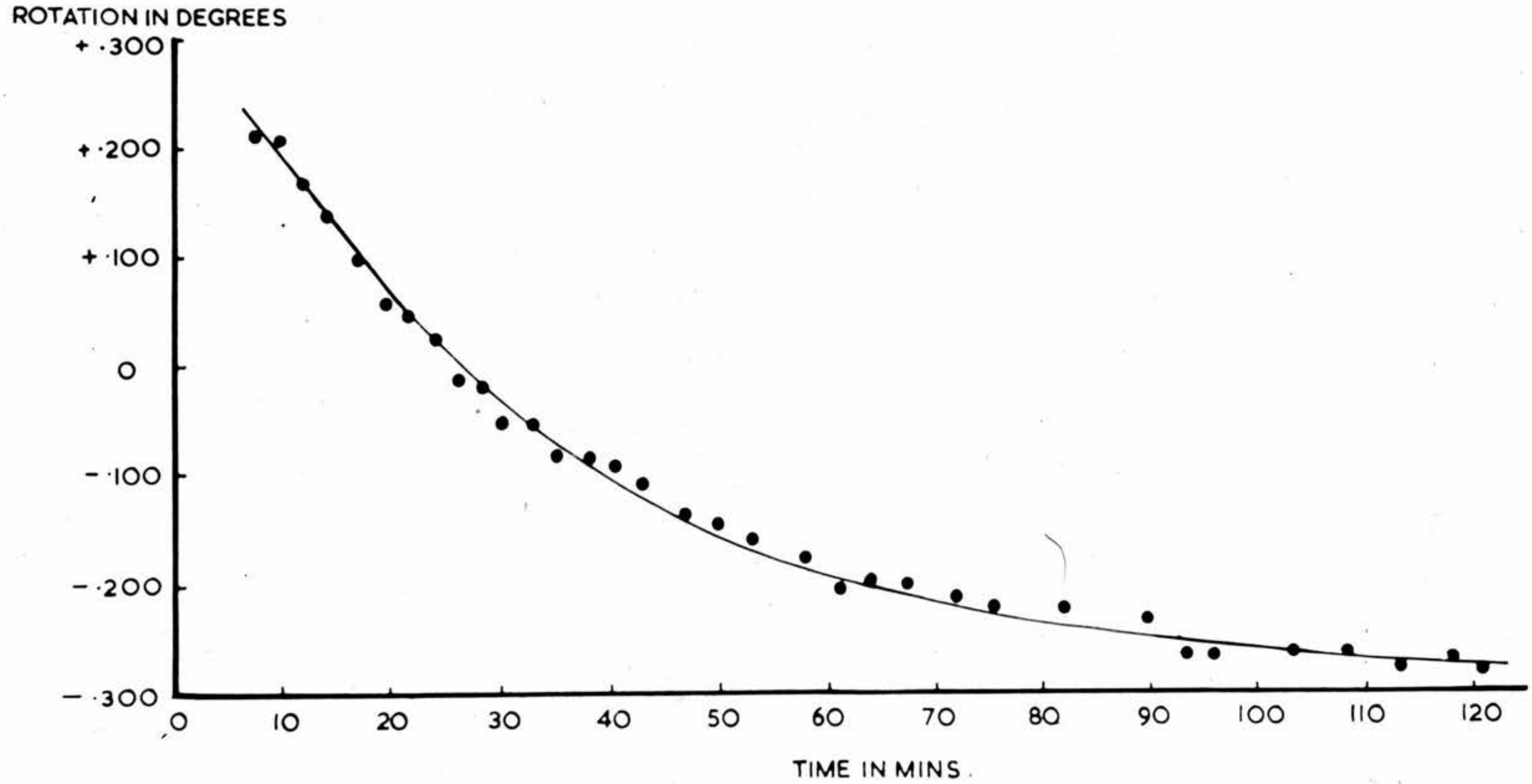
Rearrangement/

Rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β in alkaline aqueous solution

Marrian, Watson & Panattoni (1957) found that sodium borohydride reduction of the KC-5 isolated from urine yielded roughly a 1:1 mixture of 16-epioestriol, both of which were isolated and identified. In explanation of this finding it was postulated that the isolated material might have consisted of a mixture of 16α-hydroxy-oestrone and 16-oxooestradiol-17β produced from the former by rearrangement during the phenolic separation. The presence of 16-oxooestradiol-17β in KC-5 would also account for its lower dextrorotation, when compared with that of 16α-hydroxyoestrone. With synthetic 16α-hydroxy-oestrone available, this possibility was tested by simulating the conditions of the phenolic separation step in the isolation work. It was found that after allowing a solution of 16α-hydroxyoestrone in aqueous N-NaOH to stand for 10 min. at room temperature, the recovered phenolic product showed a decrease in dextrorotation compared with that of the starting material, which/

FIG. 1, III.

RATE OF REARRANGEMENT OF 16α-HYDROXYOESTRONE TO 16OXOOESTRADIOL-17β IN 50% AQUEOUS ALCOHOLIC N-NaOH.



which would have corresponded to the rearrangement of about 20% of the latter to 16-oxooestradiol-17β. In addition, sodium borohydride reduction of the product was found to yield about 20% more '16-*epi*oestriol' than that obtained with pure 16α-hydroxyoestrone (cf. below). These findings seemed to support the view of Marrian *et al.* (1957) and to prove conclusively the correctness of this an attempt was made to isolate the laevorotatory component.

For this purpose a preliminary experiment was carried out to determine the optimum conditions for the highest yield of 16-oxooestradiol-17β. Fig. 1, III shows the rate of decrease in dextrorotation of 16α-hydroxyoestrone, and that rearrangement was almost complete after 90 min. Consequently, 16α-hydroxyoestrone was treated with aqueous sodium hydroxide for 2 hr. and it was found that the recovered phenolic product was indeed 16-oxooestradiol-17β as judged by its physical properties and those of its diacetate.

Reduction of 16α-hydroxyoestrone

Reduction of 16α-hydroxyoestrone by sodium borohydride yielded a mixture containing about 90% and 10% respectively of oestriol-like and 16-*epi*oestriol-like products. Accordingly, as with the diacetate, it would appear either that some rearrangement occurred during the reaction or that a small proportion of 17-*epi*oestriol was found. Since a higher yield (30%) of the 16-*epi*oestriol-like product was obtained after catalytic reduction with hydrogen and platinum oxide in neutral alcoholic solution - under conditions in which no rearrangement to 16-oxooestradiol-17β could possibly arise - the latter explanation seems the more probable one.

Thus, when more 16α-hydroxyoestrone was available, an attempt was made to settle this conclusively. However, while oestriol was readily isolated from the reaction mixture following sodium borohydride reduction and column chromatography, no pure crystalline material could be obtained from the '16-*epi*oestriol' fraction. Neither did acetylation of the partially purified product afford pure 16-*epi*oestriol or 17-*epi*-oestriol triacetate. Rearrangement of 16α-hydroxyoestrone/

hydroxyoestrone under the alkaline conditions was indicated.

Subsequently, catalytic reduction of 16α-hydroxyoestrone yielded a mixture of products, the composition of which was about the same as that obtained in the micro scale experiment. The cis-glycol was separated from oestriol by acetonide formation. Some difficulty was encountered with the decomposition of the crude acetonide; apparently it was fairly stable to hydrolysis with ethanolic phosphoric acid. Further treatment of the partially hydrolysed acetonide with ethanolic hydrochloric acid (cf. Sheehan, Erman & Cruickshank, 1957), which was used in the hydrolysis of 16-epioestriol acetonide, yielded 17-epioestriol after crystallization, identified by its melting point, its chromatographic behaviour in the chloroform-formamide system, which was developed by Dr Layne for the separation of 16-epioestriol and 17-epioestriol, and by its failure to give the David reaction. This result is in agreement with a recent report (Biggerstaff & Gallagher, 1957) that the minor reduction product of 16α-hydroxyoestrone diacetate on reduction with lithium aluminium hydride earlier encountered by Leeds et al. (1954) has now been shown to be 17-epioestriol.

Experimental

The melting points of 16α-hydroxyoestrone, 16-oxooestradiol-17β, oestriol and 17-*epi*oestriol were determined in sealed evacuated capillary tubes, while for other compounds, a microscope hot-stage was employed. The same thermometer was used in all determinations and the values given are uncorrected for emergent stem.

Details of paper chromatography and micro techniques not yet described in Section II, are given in Section IV.

Preparation of 16α-hydroxyoestrone diacetate.

16α-Hydroxyoestrone diacetate was prepared as described by Leeds, Fukushima & Gallagher (1954).

Oestrone enol diacetate (m.p. 147-150°) was treated with excess perbenzoic acid in benzene solution at room temperature. When the reaction was complete (determined by iodometry) the product was isolated and rearranged without purification by means of 72% perchloric acid in glacial acetic acid. The semi-crystalline product was purified by/

by filtration in benzene solution through a column of deactivated (10% w/v of water) Al_2O_3 and then by two crystallizations from ethyl acetate-n-hexane. The material had m.p. 169-172.5, and partition chromatographic analysis in the system 90% (v/v) ethanol in water:n-hexane did not reveal the presence of more than one Kober chromogen.

This material was roughly divided into two portions. One was recrystallized twice from methanol to yield flat diamond shaped plates, m.p. 172-174.5°, $[\alpha]_D^{18}$ + 145° (c, 516 in ethanol). (Found: C, 71.1; H, 6.7. Calc. for $C_{22}H_{26}O_5$: C, 71.3; H, 7.1%).

The other portion was recrystallized once from benzene-hexane and once from methanol. For analysis a small portion of this was recrystallized twice more from methanol. The product had m.p. 172-174.5°, $[\alpha]_D^{18}$ + 126° (c, 502 in chloroform), $[\alpha]_D^{18}$ + 153° (c, 0.503 in ethanol). (Found: C, 71.2; H, 6.8%).

For subsequent preparative work fairly pure diacetate was obtained from the rearranged product by repeated recrystallizations from methanol.

Reduction/

Reduction of 16α-hydroxyoestrone diacetate by sodium borohydride.

The reduction of 16α-hydroxyoestrone diacetate by $NaBH_4$ was studied in a series of experiments in which samples of the diacetate which had been recrystallized in different ways were allowed to react at room temperature with varying amounts of the reductant for varying times. After hot alkaline hydrolysis the reaction mixtures were diluted with water, acidified with HCl and extracted with ether. The ether extracts were washed with 8.5% (w/v) $NaHCO_3$ and with water and evaporated to dryness. Reduction was usually complete under these conditions, as was indicated by the absence of Kober chromogen in the ketonic fraction after Girard separation.

About 50 μg. of each product was analysed by partition chromatography with the system 70% (v/v) methanol in water: ethylene dichloride, and in each case the presence of two different Kober chromogens chromatographically indistinguishable from oestriol and 16-*epi*oestriol respectively was detected. The more 'polar' Kober chromogen was isolated in one experiment and identified as/

as oestriol by a mixed m.p. with authentic oestriol. Owing to lack of material conclusive evidence concerning the nature of the less 'polar' Kober chromogen was not obtained. The results of these experiments are summarized in Table 1.IIIA.

Table 1.IIIA. Reduction of 16α-hydroxyoestrone diacetate by sodium borohydride in methanolic solution: relative proportions of oestriol and '16-*epi*oestriol' in the products after alkaline hydrolysis.

Wt. of diacetate (mg.)	Purification method	Wt. of $NaBH_4$ (mg.)	Reduction time (min.)	Composition of hydrolysed reduction product (%)	
				Oestriol	'16-*epi*oestriol'
20.3	(a)	15+10	40+60	81.2	18.2
20.0	(b)	15+10	40+60	81.5	18.5
21.0	(b)	5+2	15+20	83.5	16.5
20.0	(c)	4+2.5	15+20	81.2	18.8
20.0	(c)	2.5	35	82.2	17.8
20.0	(d)	4+2	15+20	85.3	14.7
5.3	(c)	5	40	81.8	18.2

(a) Recrystallized twice from ethyl acetate-*n*-hexane

(b) Recrystallized twice from ethyl acetate-*n*-hexane, once from benzene-*n*-hexane and three times from methanol.

(c) Recrystallized twice from ethyl acetate-*n*-hexane and twice from methanol.

(d) Recrystallized twice from ethyl acetate-*n*-hexane, once from benzene-*n*-hexane and five times from methanol.

Acid hydrolysis of 16α-hydroxyoestrone diacetate.

To a solution of 170 mg. of 16α-hydroxyoestrone diacetate (m.p. 172-174.5, $[\alpha]_D^{18}$ +150° (ethanol)) in 20 ml. methanol were added 5 ml. 5N-H_2SO_4 and the solution was allowed to stand at room temperature for 5 days. After dilution with 140 ml. of ethyl acetate the solution was washed with 8.5% (w/v) $NaHCO_3$ solution and water, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The yellow crystalline residue (130 mg.) on crystallization from methanol yielded 52 mg. of colourless crystals, m.p. about 216 and 238-240.5°, $[\alpha]_D^{16}$ +180° (c, 490 in ethanol). Found: C, 75.6; H, 7.3. Calc. for $C_{18}H_{22}O_3$: C, 75.5; H, 7.7%).

A portion of this material was acetylated with acetic anhydride and pyridine at room temperature overnight. The product after crystallization from methanol had m.p. 169-173° and $[\alpha]_D^{20}$ +149° (c, 0.504 in ethanol). The melting point was not depressed after admixture either with the above described 16α-hydroxyoestrone/

oestrone diacetate (analytical sample 2) or a sample of the latter (m.p. 168.5-172°) supplied by Dr T.F. Gallagher.

16α-Hydroxyoestrone from 16α:17α:epoxyoestra-1:3:5-triene-3:17β-diol diacetate.

A mixture of 45 mg. of the epoxyacetate in 4 ml. of methanol and 1 ml. of $5N-H_2SO_4$ was allowed to stand at room temperature for 5 days. After dilution with ethyl acetate the solution was washed with 8.5% (w/v) $NaHCO_3$ and water, dried and evaporated to dryness. The product (37 mg.) yielded 11 mg. of colourless needles after crystallization from methanol. These had m.p. 237-239° (shrinkage at 215°) and $[\alpha]_D^{17}$ +174° (c, 0.500 in ethanol). Acetylation at room temperature with acetic anhydride and pyridine afforded a product which had m.p. 167-172° after one crystallization from methanol. The melting point was not depressed after admixture with 16α-hydroxyoestrone diacetate (m.p. 172-174.5°).

In large scale preparative work the crude epoxide was hydrolysed without prior purification to 16α-hydroxyoestrone.

Reduction /

Reduction of 16α-hydroxyoestrone with sodium borohydride.

To a solution of 5 mg. of 16α-hydroxyoestrone in 2.5 ml. methanol was added 5 mg. of Na borohydride. After 40 min. at room temperature the solution was diluted with water, acidified with HCl, and extracted with ether. The ether extract was washed with $NaHCO_3$ and water and evaporated to dryness. Chromatographic analysis of 50 µg. of the product showed the presence of 90 and 10% respectively of 'oestriol-like' and '16-epioestriol-like' Kober chromogens.

Catalytic reduction of 16α-hydroxyoestrone.

To a suspension of about 0.4 mg. of Adams platinum oxide catalyst in 4 ml. ethanol was added 155 µg. of 16α-hydroxyoestrone. The mixture was shaken for 30 min. under hydrogen at atmospheric pressure. After filtration the solution and washings were evaporated to dryness. A portion of the residue was treated with Girard T reagent to remove any unreduced ketonic material. The non-ketonic fraction on chromatographic analysis was found to contain 70 and 30% respectively of 'oestriol-like' and '16-epioestriol-like' Kober chromogens.

Rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β by heat.

16α-Hydroxyoestrone ($[\alpha]_D^{18}$ +170° (ethanol), 25 mg.) was introduced into a tube which was then sealed under reduced pressure (1.4 x 10^{-4} mm. Hg). The tube was immersed in an oil bath which was rapidly heated to 220°, maintained at this temperature for 5 min., and finally heated to 230° for a further 2 min. After cooling, the slightly yellow semi-crystalline product was washed several times with n-hexane and then crystallized once from methanol. The crystalline product melted at 236-238° with only very slight shrinkage at 217°, and had $[\alpha]_D^{18}$ -69°(c, 0.508 in ethanol). Acetylation at room temperature with acetic anhydride and pyridine yielded a product with m.p. 133-137°, and the mp. after admixture with authentic 16-oxooestradiol-17β diacetate (m.p. 134-137°) was 133-136.5°.

Rearrangement/

Rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β in alkaline aqueous solution.

To a solution of 10.4 mg. of 16α-hydroxyoestrone ($[\alpha]_D^{17}$ + 181° (EtOH)) in 1 ml. ethanol was added 100 ml. N-NaOH solution. After standing for 10 min. at room temperature sufficient 5N-H_2SO_4 was added to effect 90% neutralization. The pH of the solution was brought to 9 with gaseous CO_2. The mixture was extracted with ether and the ether extract after washing with 2% (w/v) $NaHCO_3$ and water and drying over anhydrous Na_2SO_4, was evaporated to dryness. The unpurified product, 10.2 mg., had $[\alpha]_D^{17}$ +122° (c, 0.50 in ethanol). The reduction products of this material with $NaBH_4$ consisted of 72.5 and 27.5% respectively of 'oestriol-like' and '16-epioestriol-like' Kober chromogens (cf. 90 and 10% for pure 16α-hydroxyoestrone). These findings suggested that some 16α-hydroxyoestrone was rearranged to 16-oxooestradiol-17β.

To confirm that this was so, an attempt was made to isolate 16-oxooestradiol-17β from the product. The following experiment was carried out to determine the conditions for obtaining an/

an optimum yield of 16-oxooestradiol-17β.

A solution of 16α-hydroxyoestrone (2.33 mg., $[\alpha]_D^{17}$ + 181°) in 0.25 ml. ethanol and 0.25 ml. 2N-NaOH was introduced into an optical rotation tube. Successive polarimetric readings were taken from $7\frac{1}{2}$ min. onwards and were discontinued at the end of 2 hours. No further change in rotation was observed from 90 min. onwards.

Rearrangement was thus complete at 2 hours. Consequently, the following further experiment was carried out.

To a solution of 48 mg. of 16α-hydroxyoestrone ($[\alpha]_D^{17}$ + 181° (ethanol)) in 2 ml. of ethanol was added 100 ml. of N-NaOH. The solution was allowed to stand at room temperature for 2 hours and then acidified with 5N-H_2SO_4. The reaction mixture was extracted with ether, and the extract, after washing with $NaHCO_3$ and water, was dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product (39.5 mg.) had $[\alpha]_D^{20}$ -83° (c, 0.493 in ethanol). After crystallization once from methanol 19 mg. of product/

product having m.p. 236.5-238.5° and $[\alpha]_D^{19}$ -95° (c, 0.492 methanol) was obtained. The acetyl derivative prepared from this material after crystallization twice from ethyl acetate-n-hexane, had m.p. 133-136°, and the m.p. after admixture with authentic 16-oxooestradiol-17β diacetate was 133-137°.

Attempted characterization of the minor $NaBH_4$ reduction product of 16α-hydroxyoestrone.

To a solution of 180 mg. of 16α-hydroxyoestrone $[\alpha]_D$ + 170° in 30 ml. methanol was added 135 mg. of Na borohydride. After 1 hour another 200 mg. were added. At the end of another hour, the solution was acidified with acetic acid, concentrated to a volume of about 10 ml. and finally diluted with water. The mixture was extracted with ether and the ether extract, after washing with $NaHCO_3$ and water, was dried over anhydrous Na_2SO_4 and evaporated to dryness. The product, 178 mg., was chromatographed on ~~a~~ 120 g. Celite, using the system 70% methanol in water: ethylene dichloride. Fractions 2-7, which contained the '16-epioestriol-like' product, were combined and evaporated to dryness/

dryness. The residue, 41.5 mg., after crystallization once from methanol-benzene, yielded 27 mg. of crystalline material, m.p. 165-170° and 195-205°. After another crystallization from methanol-benzene, 14.4 mg. of material was obtained. This again melted over a wide range. This was recombined with the material recovered from the mother liquor of the second recrystallization and the whole acetylated with acetic anhydride and pyridine. The product after crystallization twice from hexane had m.p. 128-139°. A further crystallization from methanol failed to improve the melting point (cf. 16-*epi*oestriol triacetate, 151-152°; 17-*epi*oestriol triacetate, 163°).

Identification of the minor reduction product of 16α-hydroxyoestrone.

(a) Catalytic reduction - A solution of 55.8 mg. of 16α-hydroxyoestrone in 7 ml. of ethanol was shaken for 3 hours at atmospheric pressure with 15 mg. of Adams platinum oxide catalyst in an atmosphere of hydrogen. After filtration the solution and washings were evaporated to dryness.

(b))/

(b) Acetonide formation - The well-dried residue was then dissolved in 10 ml. anhydrous acetone previously saturated with dry HCl. After 40 min. the reaction mixture was diluted with ice and water, and the aqueous phase extracted with ether. The ether extract was washed with 5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness.

(c) Separation of oestriol from the acetonide - The solid residue, 56 mg., was dissolved under reflux in 200 ml. of boiling chloroform, containing 2 ml. of ethanol. The cooled solution was extracted once with water which was discarded. It was then extracted twice with equal volume of 0.1 N-NaOH and the combined alkaline extract was extracted once with 30 ml. of chloroform, which was added to the main bulk. The chloroform solution was washed four times with 25 ml. of water, dried and evaporated to dryness. The semi-crystalline oil weighed 17 mg.

(d) Acid hydrolysis of the acetonide - The crude acetonide was dissolved in 5 ml. ethanol. After adding 1.25 ml. $M.H_3PO_4$ the mixture was heated under reflux/

reflux for 75 min. and finally allowed to stand overnight at room temperature. The following day the mixture was partitioned between ether and water. The ether phase on working up yielded 17 mg. of crude product. Crystallization from methanol yielded 8.4 mg. of colourless crystals, m.p. 222-234°. Another crystallization from methanol raised this to 243-247° (evac. sealed tube). This material had $[\alpha]_D^{15}$ + 75° (ethanol) (cf. 17-epioestriol, m.p. 235-237°; $[\alpha]_D$ + 58°). On paper chromatography it showed a spot indicating unhydrolysed acetonide plus trace of 17-epioestriol. The material was recombined and dissolved in a mixture of 5 ml. of ethanol and 1 ml. of concentrated HCl. After refluxing the solution for 2 hours the product was isolated in the manner described above. The coloured solid, 12.5 mg., was treated twice with charcoal in methanol and finally crystallized from methanol-benzene. The slightly coloured crystals weighed 7.4 mg. Partition chromatographic analysis of 50 µg. of this material in the solvent system 70% methanol in water:benzene-hexane (4:1, by vol.) showed only a 17-epioestriol-like Kober/

Kober chromogen. After another charcoal treatment and recrystallization from 70% aqueous ethanol the material had m.p. 230-232° (cf. m.p. of 17-*epi*oestriol: 236.5-237° (Prelog, Ruzicka & Wieland, 1945); 230-235° (Biggerstaff & Gallagher, 1957). It did not give the David reaction. Chromatography in the chloroform-formamide system showed that it was identical with authentic 17-*epi*oestriol in mobility.

Discussion

The preparation of 16α-hydroxyoestrone diacetate and its subsequent hydrolysis to 16α-hydroxyoestrone are straightforward reactions. Yields of the diacetate were reasonably good, provided a good preparation of perbenzoic acid was employed for the epoxidation step (cf. Wragg, 1952). A higher recovery of 16α-hydroxyoestrone might be effected by using a different solvent for crystallization.

The rearrangement of 16α-hydroxyoestrone to 16-oxooestradiol-17β on heating at or near its melting point is yet another example of the thermal behaviour characteristic of ketolic compounds/

compounds (for example, see Julian & Passler, 1932). In this instance it happens that the 17β-hydroxy-16-oxo structure is the thermodynamically more stable one.

Similarly, the rearrangement of 16α-hydroxyoestrone (and its 16β-epimer) to 16-oxo-oestradiol-17β in aqueous alkaline solution is analogous to the phenomenon observed in the alkaline hydrolysis of androst-5-ene-3β:16α-diol-17-one diacetate, the product isolated being androst-5-ene-3β:17β-diol-16-one (Cooley, Ellis, Hartley & Petrow, 1955). From such evidence as is available, it would seem that the thermodynamical stabilities of the three oestrogenic 16,17-ketols fall in the following order:- 17β-hydroxy-16-oxo $>$ 16α-hydroxy-17-oxo $>$ 16β-hydroxy-17-oxo. Since the 12β- and the 17β-hydroxyl are similar in many respects, it would seem of interest to compare the above series with the sequence established for the cholanic acid 11,12-ketols, viz., 12β-hydroxy-11-oxo $>$ 11α-hydroxy-12-oxo $>$ 12α-hydroxy-11-oxo $>$ 11β-hydroxy-12-oxo (Borgstrom & Gallagher, 1949). However, the agreement stops there. For/

For, in contrast to the cholanic acid derivative, the rearrangement to the 17β-hydroxy-16-oxo structure is irreversible (cf. Watson, 1957). The stability of the remaining and as yet undescribed ketol, namely, 16-oxooestradiol-17α, is therefore of great chemical interest.

It appears that the thermodynamically stable 17β-hydroxy-16-oxo structure is more labile to chemical oxidation (cf. Section II and copper acetate oxidation in Section IV). This may presumably be due to the hyperconjugative effect of the C-18 methyl group transmitted to C-17 via C-13. On the other hand, in 16α-hydroxyoestrone this effect on C-16, already weakened by distance, is further screened off by the C-17 oxo group.

The reduction of 16α-hydroxyoestrone diacetate by sodium borohydride is of particular interest in the light of the finding that the 16-*epi*oestriol-like product obtained in the catalytic reduction of 16α-hydroxyoestrone is indeed 17-*epi*oestriol. Moreover, while much rearrangement of 16α-hydroxyoestrone to 16-oxooestradiol/

oestradiol-17β undoubtedly occurred in the large scale sodium borohydride reduction experiment, it is believed that on a micro scale, where there is an excess of the reductant, formation of the substrate-borohydride complex (i.e. reduction) should occur rapidly and predominantly, thus leaving little free ketol for rearrangement in the alkaline solution. In consequence the latter yield might consist entirely of 17-epioestriol. This agrees well with the value (10%) reported for the yield of 17-epioestriol from the lithium aluminium hydride reduction of 16α-hydroxyoestrone diacetate (Biggerstaff & Gallagher, 1957). The conditions of the latter method of reduction seem to be not alkaline enough for either epimerization or rearrangement to occur. Thus, Fajkos (1955) reduced 3β-hydroxy-16α-bromo-androstane-17-one with lithium aluminium hydride in ether solution and obtained as the major product the 17α-epimer; use of sodium borohydride in methanolic solution caused epimerization of the 16α-bromo group. If 17-epioestriol were indeed the predominant product in the 16-epioestriol-like fraction from the/

the sodium borohydride reduction of 16α-hydroxy-oestrone diacetate, it would appear that the 16α-acetoxy group has a greater steric effect on the reduction of the C-17 oxo group than the 16α-hydroxyl. These findings seem to open up an alternative and attractive route to 17α-hydroxy oestrogens. Thus, by introducing a large substituent, e.g., benzoate, t-butyrate or halogen, at the 16α-position, reduction of the C-17 oxo group either with lithium aluminium hydride or catalytically would result in a higher yield of the 17α-epimer.

SECTION III. PREPARATION OF 16-HYDROXYOESTRONES

B. Preparation of 16β-Hydroxyoestrone and Related Compounds

Introduction

With the isolation of 16α-hydroxyoestrone from urine, the previous suggestion of Marrian, Watson & Panattoni (1957) that oestrone undergoes 16-hydroxylation with the formation of 16α-hydroxyoestrone and 16β-hydroxyoestrone which may give rise to oestriol and 16-*epi*oestriol respectively by metabolic reduction of the C-17 oxo group, receives substantial support. The presence of 16β-hydroxyoestrone in urine is therefore to be expected.

Thus, the detection of a ketonic Kober chromogen more 'polar' than 16α-hydroxyoestrone in pregnancy urine in amounts consistent with the assumption that it could be the immediate precursor of 16-*epi*oestriol, provided greater impetus to the need for synthesizing 16β-hydroxyoestrone. The availability of synthetic 16β-hydroxyoestrone would be of use in subsequent isolation work and related studies.

In/

In this section the work dealing with the preparation of 16β-hydroxyoestrone and other related compounds is described.

Results

Epimerisation of 16α-hydroxyoestrone ditosylate

Several synthetic schemes were postulated for the preparation of 16β-hydroxyoestrone, two of which were based on 16α-hydroxyoestrone as the starting material.

One of the most direct routes for the conversion of 16α-hydroxyoestrone to the 16β-epimer would be the p-toluene sulphonate-acetate exchange reaction. This has been widely employed in organic synthetic work in which a change in the configuration of a secondary hydroxyl group is desired and considered feasible. In the oestrogen field Huffman & Lott (1955a) have applied it in the preparation of oestradiol-16α from the 16β-epimer. In this instance the approach of the incoming substituent is from the unhindered α side of the molecule and hence the departure of the 16β-p-toluenesulphonate is greatly facilitated. Thus it would seem unlikely that the converse transformation could be readily and successfully effected. However, in view of the/

the interest inherent in such a reaction it was felt that an attempt might be worthwhile. For, acetolysis of an α-tosylated ketone might conceivably give rise to an α, β-unsaturated ketone as well as the α-acetoxylated derivative with the same or the opposite configuration. As the experiment was of a preliminary nature, it was decided, for simplicity's sake, to carry out the acetolysis on 16α-hydroxyoestrone ditosylate. It was evident that while the desired 16-acetate could be readily hydrolysed by acid, the hydrolysis of the 3-tosyl group would not be feasible except by saponification, under which conditions both ketolic structures should rearrange to the 17β-hydroxy-16-oxo structure.

16α-Hydroxyoestrone ditosylate, m.p. 163-4°; $[\alpha]_D^{18}$ + 80° in acetone, was prepared from 16α-hydroxyoestrone in satisfactory yields. Attempted acetolysis in acetic acid solution containing potassium acetate at room temperature afforded unchanged material. However, when the acetolysis was carried out at reflux temperature of the solution, a crystalline substance besides the starting material was eventually isolated. After recrystallization/

recrystallization the product had m.p. 157-161° and $[\alpha]_D^{16}$ + 87°, and the melting point was depressed after admixture with starting material. Its elementary analysis suggested that there was no cleavage of the 16-tosyl group. The possibility therefore arose that the starting material might have undergone rearrangement to 16-oxooestradiol-17β ditosylate or epimerization to 16β-hydroxyoestrone ditosylate. The dextrorotation of the compound seemed to preclude the former possibility. To exclude this completely, the ditosylate of 16-oxooestradiol-17β, previously described by Huffman, Lott & Tillotson (1955), was prepared. Comparison of the latter material with the isolated compound by a mixed melting point determination established their dissimilarity. In addition, the ditosylate of 16-oxooestradiol-17β had $[\alpha]$ -65° (acetone) and, in contrast to the isolated compound and 16α-hydroxyoestrone ditosylate, yielded negligible colour in the Kober reaction. There seems little doubt that the compound isolated is 16β-hydroxyoestrone ditosylate.

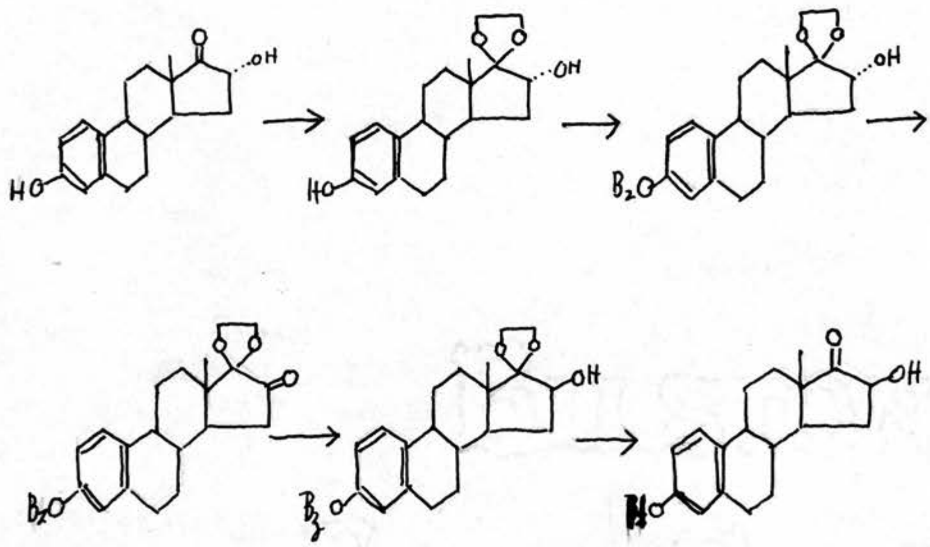
Attempted acid hydrolysis of the ditosylate was unsuccessful as might be expected. It would seem possible that cleavage of both the tosyl groups/

groups could be effected either by alkaline saponification or, better, by means of lithium aluminium hydride (cf. Schmid & Karrer (1949)), provided the 17-ketone was masked beforehand by ethylene ketal formation. The former procedure might bring about partial inversion to the 16α-ol. However, this would involve as many steps as the following route (cf. below), and was not investigated.

Conversion of the 16α-hydroxyl to the 16β-epimer via the 16-oxo derivative

Bernstein, Hiller & Stolar (1955) have prepared 16-oxo-progesterone and 16β-hydroxy- progesterone from pregn-5-ene-16α-ol-3:20-dione 3, 20-bisethylene ketal. Thus, oxidation with chromic trioxide-pyridine complex, afforded the 16-oxo derivative, which was converted to the 16β-ol on reduction with lithium aluminium hydride. Mild acid hydrolysis removed the bis-ethylene ketal groups, thereby generating the free compounds. An analogous reaction sequence with some modifications/

modifications starting from 16α-hydroxyoestrone was thus drawn up and is shown schematically below.



The protection of the phenolic group by benzoylation instead of methylation was preferred since the free compound could be more readily obtained. Acetylation of the ethylene ketal of 16α-hydroxyoestrone with acetic anhydride by the Schotten-Baumann procedure was attempted, but the yield was too low to be of practical use. The advantages of the chromic acid-pyridine complex, a mild and very useful oxidizing agent first introduced/

introduced by Poos, Arth, Beyler & Sarett (1953) into the field of steroid chemistry, have been discussed by the originators. For one thing, oxidation at position 6 of the oestrogen nucleus and, probably too, at the tertiary positions (cf. McNiven (1954) and references cited therein) encountered with the use of the chromic acid-acetic acid mixture, would be avoided. Reduction of the C-16 ketone by sodium borohydride has been shown by Huffman & Lott (1955) to yield predominantly the thermodynamically more stable 16β-epimer.

Incidentally, the availability of the ethylene ketal permitted an attempt at bringing about a change in configuration of the 16α-hydroxyl. By equilibrating the sodium complex of 16α-hydroxyoestrone-17-ethylene ketal in sodium n-butoxide solution the thermodynamically more stable 16β-epimer might be formed. However, in one experiment the starting material was largely recovered. Nevertheless, the stability of the ketal group under drastic alkaline conditions was thereby clearly demonstrated.

Ketal/

Ketal formation proceeded satisfactorily and in good yields. The ethylene ketal had m.p. 235-236.5° and $[\alpha]_D \rightarrow 0$ (ethanol). In the latter respect it is interesting to note that 17α-ethinyloestradiol-17β has $[\alpha] = +1°$; this might be expected since the steric environments around C-17 in these two compounds are essentially the same.

Benzoylation according to the Schotten-Baumann procedure also gave high yields, but, apparently owing to the state of purity of the benzoyl chloride used, the 3-benzoate obtained was seriously contaminated, as was evident from the poor carbon analysis. However, the product could be employed for the next step without ambiguous results.

Oxidation of the 3-benzoate 17-ethylene ketal proceeded smoothly. That oxidation might occur elsewhere in the nucleus seemed to be excluded, since the same product was obtained whether the reaction period was short or prolonged. The formation of a 16-oxo group was evident from the laevorotatory nature of the product. Moreover, in/

in contrast to the 16-hydroxy 17-ethylene ketals, it did not give a Kober colour, while in concentrated H_2SO_4 solution it produced nearly the same colour as given by 16-oxooestrone. The elementary analyses, though not very satisfactory, agreed with the assigned formula. The compound had m.p. 159.5-161.5°.

Reduction of the 16-oxo derivative by sodium borohydride in benzene methanolic solution yielded 16β-hydroxyoestrone-3-monobenzoate 17-ethylene ketal. The product had m.p. 190-194°. Hydrolysis of this compound as effected with 16α-hydroxyoestrone diacetate did not yield homogeneous products, as was also evident from partition chromatography. The benzoyl as well as the ketal function was difficultly cleaved under these conditions. Accordingly, in a later experiment the reduction product was saponified and the crude product further hydrolysed with acid. Complete hydrolysis of the ketal was not effected; this stability was rather unexpected. However, it was considered likely that the small amount of the ketal remaining would not interfere with the purification of the main product, i.e., 16β-hydroxyoestrone/

oestrone. Despite several attempts at fractional crystallization no sharp-melting material was obtained. The negative optical rotation of the crude product suggested rearrangement to 16-oxo-oestradiol-17β at some stage. It was not expected that some cleavage of the ketal occurred simultaneously during alkaline hydrolysis with the formation of 16β-hydroxyoestrone, which would then rearrange to 16-oxooestradiol-17β. However, the facile conversion of 16β-hydroxyoestrone to 16-oxooestradiol-17β in acid solution was not known at that time and hence these results seemed rather perplexing.

Reaction of oestrone acetate with lead tetraacetate.

The α-acetoxylation of steroidal ketones by the action of lead tetraacetate has been successfully carried out by numerous workers. For example, Reichstein & Montigel (1939) used this reaction in the preparation of 21-acetoxy-progesterone, and, more recently Sondheimer, Kaufmann, Romo, Martinez & Rosenkrantz (1953) in the preparation of 2α- and 2β-acetoxy-testosterone-17β acetate. However, prior protection/

protection of the phenolic group is necessary before α-acetoxylation of oestrone is to be attempted. For, it has been shown (Cavill, Cole, Gilhem & McHugh, 1954) that phenol, o-, p-cresol and other monophenols suffer oxidative attack of the aromatic ring; under these conditions, however, phenyl acetate does not react with lead tetraacetate. The benzene ring itself is fairly stable, as is shown by the preparation of mixed benzoins from the corresponding deoxybenzoins (Badcock, Cavill, Robertson & Whalley, 1950).

Attempted α-acetoxylation of oestrone acetate with lead tetraacetate in acetic acid solution afforded no crystalline product except starting material after alumina chromatography and crystallization. Quite an appreciable portion of the oxidation product was only eluted from alumina after using 'polar' solvent mixtures and these were presumably highly oxygenated substances.

Reaction of oestrone enol diacetate with lead tetraacetate.

At this stage of the investigation Professor W.S.Johnson of the University of Wisconsin kindly made/

made available to us a copy of his manuscript prior to publication (this has since appeared; see Johnson, Gastambide & Pappo, 1957), describing the action of lead tetraacetate on the enol acetate of *epi*androsterone acetate, which resulted in the stereoselective introduction of a 16β-acetoxy group.

The analogous reaction with oestrone enol diacetate would, as might be expected, yield 16β-hydroxyoestrone diacetate. This proved to be so. The pure product melted at 139-141° and had $[\alpha]_D^{15}$ +132° (ethanol); and was apparently the lower-melting modification of the same substance, which has been obtained in two forms by Biggerstaff & Gallagher (1957). The compound was characterized by sodium borohydride reduction, followed by alkaline hydrolysis, to yield predominantly one product, namely, 16-*epi*oestriol.

Hydrolysis of 16β-hydroxyoestrone diacetate to 16β-hydroxyoestrone.

Dilute acid hydrolysis of the 16β-acetoxy derivative of *epi*androsterone acetate resulted in rearrangement of the desired product to the 17β-hydroxy-16-oxo isomer.

It was anticipated that acid hydrolysis of 16β-hydroxyoestrone diacetate would yield 16-oxo-oestradiol-17β. A preliminary experiment, however, revealed that only partial rearrangement occurred in the oestrogen series. For the purified hydrolysed product on reacetylation yielded 16β-hydroxyoestrone diacetate, while the laevorotatory component remained in the mother liquors.

It was hoped that enzymic hydrolysis of the diacetate under similar conditions used by Mattox, Mason & Albert (1953) for the enzymic hydrolysis of aldosterone diacetate, would give a higher yield of 16β-hydroxyoestrone. However, it seemed that partial rearrangement also occurred in neutral solution, as was indicated by the low dextrorotation of the crude product. In view of the practical difficulties inherent in the enzymic method of hydrolysis, which apparently had no demonstrable advantage over acid hydrolysis, further work was discontinued.

16β-Hydroxyoestrone like its 16α-epimer apparently undergoes rearrangement to 16-oxo-oestradiol-17β near its melting point. It was thus concluded/

concluded that the melting point was not a good criterion of purity in this particular instance. Accordingly, it was decided to concentrate the 16β-hydroxyoestrone by crystallizing the crude hydrolysed product repeatedly and combining those fractions that showed high dextrorotation. The product obtained in this manner was dried to constant weight at 100° in vacuo, but it was still not analytically pure. This might be due to solvent of crystallization (cf. Biggerstaff & Gallagher, 1957). The melting point of the dried sample determined in an evacuated sealed capillary was 237-239.5°, while on a hot stage it was 218-234° (main bulk 224-227°). On partition chromatography it was found to contain about 6% of 16-oxo-oestradiol-17β.

One interesting fact emerged in the latter connection. In this and previous attempts (the preceding three synthetic schemes) the 16β-derivative was invariably hydrolysed with acid, followed by partition chromatography in the system 70% methanol/benzene-hexane (4:1, by vol.). The elution pattern of the KC-5 fraction suggested homogeneity/

homogeneity, and in view of the belief that 16β-hydroxyoestrone might be more 'polar' than KC-5 (cf. Appendix I), the conclusion was made that the desired product was not obtained. It therefore appeared that with ~~a~~ certain proportions of 16β-hydroxyoestrone and 16-oxooestradiol-17β present (e.g. in the enzymically hydrolysed product) resolution in this system was not possible, while this could be achieved with the highly concentrated product (cf. above).

Experimental

Unless otherwise stated, all melting point determinations were carried out on a microscope hot-stage.

Preparation of 16α-hydroxyoestrone ditosylate

16α-Hydroxyoestrone ($[\alpha]_D^{16}$ = +178° (EtOH); 20 mg.) was treated with 100 mg. p-toluene sulphonyl chloride in 0.5 ml. anhydrous pyridine at 0° for 1 hour. After another 23 hours at room temperature the excess reagent was decomposed with crushed ice and water. The precipitate was filtered, washed with water and dried over H_2SO_4 in vacuo. The crude product after treatment with charcoal in acetone-methanol yielded on crystallization 20 mg. of crystalline material, m.p. 158-164°. Another crystallization from acetone-methanol (1:3, by vol.) at 5° gave colourless prisms, m.p. 163-164° and $[\alpha]_D^{17.5}$ + 75° (c, 0.521 in acetone). (Found: C, 64.45; H, 5,53. Calc. for $C_{32}H_{34}O_7S_2$: C, 64.62; H, 5.76%). It gave a strong Kober colour.

Attempted/

Attempted acetolysis of 16α-hydroxyoestrone ditosylate.

(a) At room temperature - 16α-Hydroxyoestrone ditosylate was prepared from 60 mg. of 16α-hydroxyoestrone in the manner described above.

A solution of 115 mg. of the unpurified ditosylate in 2.5 ml. glacial acetic acid containing a few drops of acetic anhydride was shaken occasionally with 100 mg. freshly fused potassium acetate for 32 hours at room temperature. The reaction mixture was then thoroughly chilled, partially neutralized with alkaline solution equivalent to 2 ml. of the acetic acid and extracted with ether. The combined ether extract was washed with $NaHCO_3$ and water and evaporated to dryness. The coloured residue dissolved in acetone was treated with charcoal and finally recrystallized twice from acetone-methanol. The product melted at 163.5-165° and showed $[\alpha]_D^{18}$ + 86° (c, .502 in acetone). (Found: C, 64.80; H, 5.47%). When mixed with authentic 16α-hydroxyoestrone ditosylate,no melting point depression was observed.

(b) /

(b) At reflux temperature - (i) A solution of 45 mg. of the ditosylate, m.p. 150-160° in 4 ml. acetic acid and 0.5 ml. acetic anhydride with 30 mg. freshly fused sodium acetate in suspension was refluxed for 1 hour. Fractional crystallization of the product worked up in the usual way afforded mainly the starting material, m.p. 155-159°. From the mother liquors of the first crystallization colourless needles, contaminated with some ditosylate (prisms) separated out on standing, 3 mg., m.p. 145-151°, sintering from 137°. Two crystallizations from methanol did not improve the melting point.

(ii) Another experiment with 90 mg. of the ditosylate and corresponding amounts of the other reactants yielded 77 mg. of product. This on chromatography on deactivated alumina and fractional crystallization afforded 13 mg. of material, m.p. 142-145° with previous sintering.

(iii) A longer reaction time was tried. A solution of 250 mg. unpurified ditosylate, prepared from 124 mg. 16α-hydroxyoestrone, in 16 ml. acetic acid and 2 ml. acetic anhydride was heated under reflux with 130 mg. freshly fused/

fused sodium acetate. After 7 hours, excess acetic acid was removed under reduced pressure. The liquid residue was diluted with ice water and extracted with benzene. The combined benzene extract was washed with $NaHCO_3$ and water and evaporated to dryness under reduced pressure. The oily residue, 235 mg., failed to crystallize and was finally chromatographed on acid-washed alumina (7 g.; activity II/III). Elution was carried out with benzene-hexane mixtures, benzene, and benzene-ether. The mixture benzene-hexane (1:1, by vol.) eluted some 16α-hydroxyoestrone ditosylate and was not investigated. The desired product was eluted with a (3:2, by vol.) benzene-hexane mixture and benzene. The combined fractions afforded 96 mg. of semi-crystalline oil, which, after three recrystallizations from ethyl acetate-methanol, yielded the pure product, slender needles, m.p. 157-161°, $[\alpha]_D^{16}$ +87° (c, 4% in acetone). (Found: C, 64.81; H, 5.69. Calc. for $C_{32}H_{34}O_7S_2$: C, 64.62; H, 5.76%). When mixed with authentic 16α-hydroxyoestrone ditosylate (m.p. 163-164°), the melting point was 137-157°.

It/

It gave a Kober colour.

To exclude the possibility that this might be 16-oxooestradiol-17β ditosylate, the preparation of 16-oxooestradiol-17β ditosylate was carried out.

The crude ditosylate (60 mg.) after recrystallization twice from ethanol and twice from acetone-methanol, yielded 30 mg. of material, m.p. 159-161°, $[\alpha]_D^{17}$ -65° (c, 0.785 in acetone). (Found: C, 64.80; H, 5.74%). This depressed the melting point of the product described above. In contrast to the ditosylate of 16α-hydroxyoestrone, it did not give a Kober colour.

Preparation of 16α-hydroxyoestrone-17-ethylene ketal.

A mixture of 100 mg. 16α-hydroxyoestrone, 1.2 ml. ethylene glycol and 20 mg. of p-toluene sulphonic acid monohydrate in 60 ml. benzene was slowly distilled during one hour to remove any moisture present. The mixture (about 50 ml.) was then refluxed for $3\frac{1}{2}$ hours and finally another 10 ml. of benzene was allowed to distil over in half an hour. The cooled reaction mixture was diluted/

diluted with ethyl acetate, washed with $NaHCO_3$ and water and dried over anhydrous Na_2SO_4. The residue obtained after removal of the solvents weighed 116 mg. and was crystallized once from ethanol and then from methanol. The product, 11.5 mg., had m.p. 235-236.5° and $[\alpha]_D^{16} \rightarrow 0$ (Found: C, 73.24; H, 7.87. Calc. for $C_{20}H_{26}O_4$: C, 72.71; H, 7.87%).
From the mother liquors another 60 mg. was recovered, m.p. 234-235°.

Attempted epimerization of 16α-hydroxyoestrone ethylene ketal.

To a refluxing solution of 40 mg. of 16α-hydroxyoestrone ethylene ketal, m.p. 234-235°, in 5 ml. butanol (purified according to Clarke et al., J. Chem. Soc., 1927, 2152), metallic sodium was added till none would dissolve further. This took about an hour. The pale yellow solution was heated under reflux for another 3½ hours. After cooling the solution was diluted with 50 ml. of cold water and extracted twice with 20 ml. of ether. The aqueous/

aqueous phase was saturated with CO_2 and extracted three times with 40 ml. of ether. The combined extract was washed with $NaHCO_3$ and water and evaporated to dryness, and the semi-crystalline residue, 34.5 mg., was crystallized once from methanol-benzene, yielding 21 mg. of material, m.p. 222-235°. This was recrystallized from 80% aqueous methanol. 16α-Hydroxyoestrone ethylene ketal (16 mg.) was thus recovered, m.p. 233-234°. When mixed with the authentic specimen, the m.p. was 231-236°. No crystalline material other than impure 16α-hydroxyoestrone ethylene ketal was obtained from the mother liquors.

Benzoylation of 16α-hydroxyoestrone-17-ethylene ketal.

The ethylene ketal (m.p. 233-236°; 75 mg.) was dissolved with warming in a mixture of acetone (2 ml.) and NaOH solution (25 ml.; 0.045 equivalent). One ml. of freshly redistilled benzoyl chloride was added and the mixture shaken vigorously. After 5 min. the procedure was repeated. At the end of 10 min. the solution was extracted with ethyl/

ethyl acetate. The organic phase was repeatedly washed with $NaHCO_3$ and then with water. The crude product obtained after removal of the solvent weighed 106 mg. This was suspended in 4 ml. petroleum ether (b.p. 60-80°) and dissolved under reflux by the addition of benzene (1 ml.). On cooling 65 mg. of leafy platelets were obtained, m.p. 151-157°. Two more crystallizations from benzene-petroleum ether afforded 23 mg. of material, m.p. 161-164°. (Found: C, 73.1; H, 6.8. Calc. for $C_{27}H_{30}O_5$: C, 74.6; H, 6.9%).

Oxidation of 16α-hydroxyoestrone 3-monobenzoate 17-ethylene ketal with CrO_3

To a solution of the ketal benzoate (80 mg.) in 1 ml. of pyridine was added a slurry of the chromic-trioxide-pyridine complex, prepared beforehand by adding slowly 77 mg. of CrO_3 to 0.8 ml. of pyridine. After $17\frac{1}{2}$ hours at room temperature the dark red mixture was diluted with 30 ml. of water and extracted twice with 50 ml. of benzene-ether (1:1, by vol.) and finally once with 50 ml. of benzene. The combined extracts were washed with $NaHCO_3$ and water, and evaporated to/

to dryness. 71 Mg. of crude product was obtained. After one crystallization from methanol-benzene (4:1, by vol.) the 16-oxo derivative melted at 158-160°. Two more recrystallizations from methanol-benzene raised the m.p. to 159.5-161.5°. When mixed with the 16α-hydroxy compound (m.p. 158-160°) the melting point was 140-145°. The product had $[\alpha]_D^{17}$ = -73° (ethyl acetate). (Found: C, 74.7; H, 6.5. Calc. for $C_{27}H_{28}O_5$: C, 75.0; H, 6.5%). It gave an immediate red colour with concentrated H_2SO_4. The same product was also obtained in comparable yields with a reaction time of 2 hours.

Reduction of 16-oxooestrone-3-monobenzoate-17-ethylene-ketal with $NaBH_4$.

To a solution of the 16-oxo derivative (94 mg., m.p. 155-162°) in 3 ml. of benzene and 5 ml. of methanol was added 66 mg. of sodium borohydride. After 15 min. another 58 mg. was added. At the end of another 30 min. the reaction mixture was treated with 0.25 ml. acetone. It was then diluted with benzene and water. The organic phase/

phase was separated, washed with $NaHCO_3$ and water, dried and evaporated to dryness. The oily residue was leached with methanol and the crystalline material thus obtained weighed 69 mg. This was crystallized three times from benzene-petroleum ether (b.p. 60-80°) and finally once from ethyl acetate-methanol. The product had m.p. 190-194°. (Found: C, 74.1; H, 6.8. Calc. for $C_{27}H_{30}O_5$: C, 74.6; H, 6.9).

Attempted hydrolysis of 16β-hydroxyoestrone 17-ethylene ketal 3-monobenzoate.

(i)16β-Hydroxyoestrone-3-monobenzoate 17-ethylene ketal, 30 mg., was dissolved in a mixture of methanol (3.5 ml.) and benzene (1 ml.). After adding 0.5 ml. 10 N-H_2SO_4 the solution was allowed to stand at room temperature for 5 days. Some solid separated out after 2 days. The product was isolated in the manner as described for the acid hydrolysis of 16α-hydroxyoestrone di-acetate. A portion of this was chromatographed on Celite with the system 70% methanol in water: benzene-hexane (4:1 by vol.). Analysis showed the presence of about 2% of unhydrolysed benzoate (with or without the ketal grouping, 28% of the ketal/

ketal (16β-hydroxy) and 70% of a 16α-hydroxy-oestrone-like component.

(ii) In the following experiment, 72 mg. of the oxo-derivative was reduced with 140 mg. of $NaBH_4$ in a mixture of 3 ml. of benzene and 7 ml. of methanol. At the end of one hour, 0.6 g. of KOH was added to the reaction mixture. After standing overnight at room temperature, excess methanol was removed under reduced pressure and the liquid residue diluted with water. The solution was brought to pH 8.4 with CO_2 and then extracted with ether. The ether extract gave on evaporation 51 mg. of product, m.p. 190-210°, sintering from 180°. This was hydrolysed in a mixture of 2 ml. methanol and 0.5 ml. 10 N-H_2SO_4 for 4 days. 48 mg. of product was obtained, m.p. 205-215°, sintering at 188°. Crystallization with methanol failed to yield pure crystalline material. A portion of this crude product showed a slightly negative optical rotation.

Attempted/

Attempted α-acetoxylation of oestrone acetate (cf. Schneider, J. biol. Chem. 1952, 199, 235).

Acetylation of oestrone (540 mg.) with 2.5 ml. acetic anhydride in 3 ml. pyridine yielded after one crystallization from aqueous methanol 503 mg. of the acetate, m.p. 125.5-127°.

To a solution of oestrone acetate (95 mg.) in 2.8 ml. acetic acid and 1 ml. acetic anhydride at 70° was added 210 mg. of lead tetraacetate. After 24 hours the yellow solution was cooled and poured into ice and water. The mixture was extracted with ethyl acetate. The extract was washed till neutral with $NaHCO_3$ and then with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue, 112 mg., was a semi-crystalline yellow oil. Attempted purification by fractional crystallization from ethyl acetate-n-hexane was unsuccessful and the combined material was finally chromatographed on acid-washed alumina (3 g.). A (1:1, by vol.) benzene-petroleum ether(b.p. 60-80°) mixture eluted 59 mg. of semi-crystalline oil, from which was obtained after two crystallizations from aqueous methanol 25 mg. of oestrone acetate, m.p. 127-129°. This was/

was confirmed by a mixed melting point determination with authentic material (m.p. 125-128°). From the fractions eluted with increasing concentrations of benzene in hexane, benzene, benzene-methanol, only small amounts of oil were obtained.

Preparation of 16β-hydroxyoestrone diacetate.

Oestrone enol diacetate (264 mg., m.p. 138-148°) was dissolved in a mixture of acetic acid (6 ml.) and acetic anhydride (0.4 ml.). After adding 400 mg. of lead tetraacetate the reaction vessel was stoppered and placed in a thermostatically controlled oven set at 70°. After $5\frac{1}{2}$ hours the reaction mixture was cooled and then poured into ice and water. The precipitate was filtered off, washed thoroughly with water and dried in vacuo over $CaCl_2$ and KOH. Four crystallizations of the coloured product from methanol afforded 32 mg. of analytically pure material, fine needles, m.p. 139-141°, $[\alpha]_D^{15}$ +132° (c, 0.494 in ethanol). (Found: C, 71.5; 71.6; H, 7.0; 7.1. Calc. for $C_{22}H_{26}O_5$: C, 71.3; H, 7.1%). When mixed with a similar preparation supplied/

supplied to us by Professor Johnson, University of Wisconsin (m.p. 136-140°, our determination) the melting point was 137.5-140°.

In a preliminary preparation the product was extracted with ethyl acetate instead. After three crystallizations from methanol, 133 mg. of fairly pure material was obtained (Yield, 50% m.p. 131-133°). For analysis the substance was recrystallized twice more from methanol, m.p. 135-138°, $[\alpha]_D^{17}$ +129° (c, 0.492 in ethanol). (Found: C, 71.2; H, 7.5%).

The diacetate gave a full Kober colour. After reduction with $NaBH_4$ and subsequent alkaline hydrolysis the nonketonic product was chromatographed on a micro Celite column (system: 70% methanol in water:ethylene dichloride). One major product, namely, 16-*epi*oestriol, was obtained. It was further identified by its ability to give the David reaction. There was trace of an 'oestriol-like' Kober chromogen.

In the blue tetrazolium reaction 16β-hydroxyoestrone diacetate showed a reducing power nearly as great as 16-oxooestradiol-17β diacetate. In /

In both column and paper chromatography it moved slower than 16α-hydroxyoestrone diacetate.

Enzymic hydrolysis of 16β-hydroxyoestrone diacetate.

(a) Assay of lipase activity.

A rather old batch of lipase preparation (Light & Co.) was used in this preliminary experiment. Its activity was determined by the method of Huggins & Lapides (1947). Briefly, the procedure was as follows: To a mixture of 2 ml. M/15 phosphate buffer, pH 7.0, 5 ml. water and x ml. of enzyme solution (100 mg. of lipase in 100 ml. water; placed in bath at 25° for 15 min. before use) kept at 25° was added 2 ml. of p-nitrophenyl acetate solution which was adjusted to 0.333 μmol/ml. The final volume of each tube was 10 ml. and each test was carried out in duplicate.

A control with no enzyme added was set up. After 20 min. the tests and the control were measured against a distilled water blank at 400 mμ. and the corrected optical densities read against a calibration curve of p-nitrophenol in phosphate buffer, pH 7.0. The enzymic concentration/

concentration - activity curve was linear over the range investigated (0.05-2 ml. enzyme solution).

The acetyl esterase activity of the lipase preparation was found to be 0.027 μmol./min./ml. enzyme solution (cf. Mattox, Mason & Albert (1953) used o-nitrophenol acetate as the substrate).

(b) Enzymic hydrolysis.

16β-Hydroxyoestrone diacetate (6.8 mg.) was dissolved in a mixture of 30 ml. methanol and 210 ml. M/15 phosphate buffer, pH 7. The mixture was turbid but not much solid material separated out. To the mixture cooled to 30° was added 1.8 g. lipase in 60 ml. buffer. After thorough mixing the incubation mixture was set aside at 25° with occasional shaking. At the end of 66 hours the enzymic activity was found to have decreased to two-thirds of its initial value. The pH of the mixture was practically unchanged.

The cooled mixture was extracted twice with 200 ml. chloroform. Serious emulsions occurred at the interface. As much as possible of the organic phase was separated, washed once with 40 ml. of water, dried over anhydrous Na_2SO_4 and the chloroform/

chloroform removed under reduced pressure at about 50°.

The residue weighed about 40 mg. and apparently contained contaminants from the lipase preparation. This was leached twice with hexane, which was filtered. The material remaining in the flask and the filter was taken up in methanol. Removal of solvent yielded 4.4 mg. of solid.

A Kober reaction carried out on 22 μg. of this material showed the presence of 16 μg. of 16β-hydroxyoestrone. Partition chromatography in the system 70% methanol-benzene-hexane (4:1, by vol.) revealed that all the Kober chromogens were accounted for in the KC-5 (see Section II) fraction. The solid therefore contained about 3.2 mg. of 16β-hydroxyoestrone.

Assuming that the contaminants had no optical rotatory power, the specific rotation of the unpurified 16β-hydroxyoestrone in ethanol was found to be +136°.

Acid/

Acid hydrolysis of 16β-hydroxyoestrone diacetate.

(a) Preliminary experiment.

A solution of 16β-hydroxyoestrone diacetate (55 mg., m.p. 138-140°) in a mixture of 6 ml. methanol and 1 ml. 5 N-H_2SO_4 was allowed to stand at room temperature for 4 days. On working up in the manner as described for 16α-hydroxy-oestrone, 45 mg. of colourless product was obtained, m.p. 220-235°. One crystallization from methanol at -20° afforded 11 mg. of crystals (I), m.p. 215-223°, partially resolidifying at ca. 230° and finally melting at 236-239° (evac. sealed tube). This had $[\alpha]_D$ +128° (c, 0.472 in ethanol). When mixed with authentic 16α-hydroxyoestrone, the m.p. were 186-200° and 236-238°. On concentration of the mother liquor another 6 mg. was obtained (II).

Acetylation of this material in the usual way yielded 11.8 mg. of crude product. After one crystallization from methanol, 6.6 mg. of material having m.p. 135-138.5°, sintering from 128°, was obtained. Another crystallization from ethyl acetate-n-hexane afforded 3.5 mg. of pure 16β-hydroxyoestrone diacetate, m.p. 137.5-139°. When mixed/

mixed with authentic material, m.p. 135-138°, the melting point was 135-139°. The material recovered from the mother liquor of the first crystallization had $[\alpha]_D^{13}$ +65° (ethanol).

Subsequently, Grade II material examined chromatographically in the $CHCl_3/HCONH_2$ system was found to contain 2-3% of 16-oxooestradiol-17β.

(b) Second experiment.

16β-Hydroxyoestrone diacetate (117 mg., m.p. 138-140°) was hydrolysed in acid methanol in the usual way. The crude product after crystallization from methanol-benzene at 5° yielded 28 mg. of material, m.p. 235.5-238°, shrinkage at <u>ca</u> 222°, and $[\alpha]_D^{16}$ +135° (ethanol).

The material from the mother liquor was recrystallized from aqueous ethanol. After 24 hours 3.0 mg. of material separated out, $[\alpha]_D^{17}$ +165° (ethanol). On concentration another crop of crystals was obtained, 24 mg. This was recrystallized twice from methanol-benzene and the product, 18.2 mg., had $[\alpha]_D^{16}$ +180° (ethanol). The material, 30.2 mg., recovered from the combined mother liquors showed $[\alpha]_D^{17}$ +149° (ethanol)/

(ethanol).

The two batches of crystalline material with higher dextrorotation (viz. +165°; +180°) were combined and recrystallized once from methanol-benzene to yield 12.8 mg. of product. For analysis, this was dried to constant weight at 100° in vacuo. (Found: C, 78.5; H, 7.8. $C_{18}H_{22}O_3$ requires C, 75.5; H, 7.7%). Chromatographic analysis on a micro column showed ca 6% of 16-oxooestradiol-17β and 94% of 16β-hydroxyoestrone.

The remaining material recovered from the mother liquors was recrystallized twice from ethanol. The product, 25 mg., was slightly contaminated with 16-oxooestradiol-17β (3-4%; paper chromatography in the $CHCl_3$-$HCONH_2$ system).

Discussion

The epimerization of 16α-hydroxyoestrone ditosylate in acetic acid solution in the presence of acetate ion is an example in the oestrogen series of the interesting stereochemistry of C-16. Thus, Johnson, Gastambide & Pappo (1957) have shown/

shown that on equilibrating either the 16α- or the 16β-acetoxy derivative of _epi_androsterone acetate in potassium acetate - acetic acid solution for 16-45 hr., a 1:1 mixture of the two 16-epimers was obtained. Similarly, Fajkos (1955) found that even in relatively mild acid conditions 3β-acetoxy-16α-bromoandrostane-17-one was converted to its 16β-epimer. Epimerization at C-16 also occurred when the 16α-bromo-17-ketone was reduced with sodium borohydride in methanolic solution. He ascribed this ease of epimerization to the tendency of a bulky and negative substituent to assume a more favourable configuration, i.e. 16β. However, more recently Ellis, Patel & Petrow (1958) found that 3β-acetoxy-16α-bromo-androst-5-ene-17-one did not undergo epimerization under the conditions of acid hydrolysis. Neither was there evidence for the concomitant formation of the 16β-epimer during brief Oppenauer oxidation of 16α-bromo-3β-hydroxyandrost-5-ene-17-one. Similar findings were obtained with 16α-chloro-testosterone, though on prolonged Oppenauer oxidation epimerization did occur. It therefore appears/

appears that the epimerization of a 16α-substituent is rather dependent on the degree of saturation in either ring A or B.

The stability of the 16-tosyloxy group to acid treatment is not surprising since aliphatic tosylates, like the aryl tosylates, are hydrolysed only under alkaline conditions. The methanolysis of the 3β-tosyl group of dehydroepiandrosterone 3β-tosylate (cf. Fajkos, 1955) is generally attributed to the activating effect of the adjacent double bond at position 5, while the solvolysis of the 3β-tosyl group of β-cholestanyl tosylate in methanol or tertiary butyl alcohol (Pappas, Meschino, Fournier & Nace, 1956) is less readily explicable.

The second synthetic scheme involving oxidation of the 16α-hydroxyl group of 16α-hydroxyoestrone proved promising and had it not been for the facile character of the 16β-hydroxy-17-oxo structure in acid solution, isolation of free 16β-hydroxyoestrone might have been feasible.

The failure to effect α-acetoxylation of oestrone acetate with lead tetraacetate in acetic acid solution may be attributed to the comparatively inert/

inert nature of the methylene group at C-16. On the other hand, the lability of the bond between C-13 and C-17 to peracids is well documented in the literature (cf. Jacobsen, 1947). Though no attempts have been made to identify the more 'polar' components among the lead tetraacetate oxidation product of oestrone acetate, it would not be surprising to find that one of these might be the 13, 17-*seco*-lactone.

The above schemes (except that of α-acetoxylation of oestrone acetate) would of course appear too long and laborious when a two-step synthesis from oestrone enol diacetate became available. Moreover, the latter method gave encouragingly good yields too.

The facile rearrangement of 16β-hydroxyoestrone to 16-oxooestradiol-17β deserves further comment. This implies that 16β-hydroxyoestrone is just as susceptible as 16-oxooestradiol-17β to destruction under alkaline conditions. It might therefore be expected that in contrast to its 16α-epimer its blue tetrazolium reducing capacity would be nearly equal to that of 16-oxooestradiol-17β. This has since been shown to be the case. Moreover, the ease with which it rearranges/

rearranges to 16-oxooestradiol-17β in alkali, hitherto suspected, has since been elegantly demonstrated by Dr Layne. By means of the chloroform-formamide system, which effects the separation of the three ketols, it was shown that 10 min. exposure to alkali (under these conditions 16α-hydroxyoestrone was only about 20% isomerized) resulted in complete rearrangement of 16β-hydroxyoestrone to 16-oxooestradiol-17β.

It also appears that some rearrangement of 16β-hydroxyoestrone had occurred in neutral alcoholic solution, since the analytical sample obtained from concentrates of high dextrorotation material (i.e. +165; +180°; cf. reported value for pure 16β-hydroxyoestrone + 173.7° (ethanol) in Biggerstaff & Gallagher (1957)) was eventually found to contain about 6% of 16-oxooestradiol-17β.

Finally, the partial rearrangement of 16β-hydroxyoestrone to 16-oxooestradiol-17β in acid solution appears at first sight rather surprising in view of the findings of Johnson <u>et al</u>. (1957). It remains to be seen whether this behaviour, like the epimerization of a 16α-substituent, is linked to certain structural features of the steroid nucleus.

SECTION IV. ISOLATION OF 18-HYDROXYOESTRONE FROM THE URINE OF PREGNANT WOMEN

Introduction

In the course of chromatographic analysis of ether-soluble phenolic-ketonic extracts from enzymically hydrolysed urines some Kober chromogenic material was detected in fractions slightly more 'polar' than 16α-hydroxyoestrone (KC-5). In view of the large amounts of KC-5 present (ca. 200 μg./100 ml. urine) the possibility existed that this 2-3 μg. of Kober chromogenic material might be KC-5 itself. The possibility that 16-*epi*oestriol, which has similar chromatographic properties in the solvent system used, had leaked into the ketonic fraction and thus become confused with this new Kober chromogen, had also to be examined. By subjecting several crude fractions to another Girard separation and re-chromatographing the ketonic fraction, nearly 60% of the starting material was recovered unchanged. Thus it was clearly shown that a sixth Kober chromogen (KC-6) occurred in pregnancy urine.

The/

The present section describes the isolation of an oestrogen derivative (KC-6A) from the KC-6 fraction and its identification as 18-hydroxy-oestrone. Evidence is also available on the presence of another Kober chromogen (KC-6B) in the same fraction which has since been obtained in crystalline form. Characterization studies on KC-6B are in progress.

Results

Preliminary investigations on KC-6

By methods similar to those employed in the isolation of KC-5 (Marrian, Watson & Panattoni, 1957) concentrates of KC-6 were obtained. While waiting for the accumulation of material sufficient for isolation work, preliminary investigations were carried out with such concentrates in the hope of obtaining some information on the probable structure of this new Kober chromogen.

In view of the finding that the amount of 16α-hydroxyoestrone in pregnancy urine was roughly sixty times that of KC-6 - the same relationship holds/

holds for oestriol and 16-*epi*oestriol - it was thought that KC-6 might be 16β-hydroxyoestrone, the hypothetical precursor of 16-*epi*oestriol. Moreover, in the blue tetrazolium reaction of Mader & Buck (1952) crude KC-6 fractions showed a reducing power nearly equal to that of an equivalent amount of 16-oxooestradiol-17β.

The behaviour of KC-6 in strongly alkaline solutions was then studied since both 16α-hydroxyoestrone and 16-oxooestradiol-17β, by virtue of their α-ketolic groupings, were completely destroyed by N-NaOH at room temperature in 15 hr. Under these conditions, however, KC-6 underwent only 15-20% destruction.

Likewise, KC-6 was found to be relatively stable towards ether peroxides. In an experiment in which each compound was refluxed in impure ether for 30 min., the recoveries of KC-6, 16α-hydroxyoestrone and 16-oxooestradiol-17β were 75, 60 and 40% respectively.

Reduction of KC-6 with Na borohydride

On reduction with sodium borohydride KC-6 yielded a 1:1 mixture of two products which behaved/

behaved like 16-_epi_oestriol and oestriol on chromatography in the system 70% methanol in water:ethylene dichloride. In the David reaction the 16-_epi_oestriol-like component gave a transient greenish-blue colour while the oestriol-like component gave a blue colour. The David reaction on KC-6 itself was negative.

Sodium borohydride reduction of the crude KC-6 acetate (see below) followed by alkaline hydrolysis yielded a 3:2 mixture of 16-_epi_oestriol-like and oestriol-like compounds. The David colours were again weak.

On methylation of the reduction products with dimethyl sulphate in alkaline solution and chromatography of the methylated products in the solvent system 70% methanol in water:benzene-hexane (1:1, by vol.) a novel interesting fact was revealed. Not only was the 3-methyl ether of the oestriol-like compound more 'polar' than the 3-methyl ether of oestriol, but also its elution pattern seemed to indicate the presence of more than one Kober chromogen. The less 'polar' methyl ether was indistinguishable chromatographically from that of 16-_epi_oestriol (and 17-_epi_oestriol).

Catalytic/

Catalytic reduction of KC-6

Catalytic reduction was attempted by passing a stream of hydrogen through a neutral alcoholic solution of KC-6 using a suspension of pre-reduced platinum oxide as catalyst. While 16-oxo-oestradiol-17β was reduced to the extent of 30% in 2 hr., only 3% of KC-6 was reduced. In the Tower micro-hydrogenation apparatus KC-6 yielded mainly 18.5% of the 16-*epi*oestriol-like compound.

It appeared to us that this resistance towards catalytic hydrogenation might be due to a cyclic hemiacetal structure - a feature commonly shown by α-ketols in solution. Since reduction with sodium borohydride was effected quantitatively, it seemed that KC-6 should be more susceptable to catalytic reduction in alkaline solution. Accordingly, reduction was attempted in N-NaOH alcoholic solution. The reduction though still incomplete after 5 hr. (20-25% recovered in the ketonic fraction) was about 2.5 times that achieved in neutral alcohol. The ratio of the 16-*epi*oestriol- and oestriol-like products was about 1:2, whereas in a parallel $NaBH_4$ reduction the/

the ratio was 3:2. Surprisingly, no David colour was obtained with the oestriol-like product.

These results were not inconsistent with the presence of an α-ketolic grouping in KC-6.

<u>Formation of an oestrone-like compound by KC-6 in acid methanol</u>

One of the most interesting observations on KC-6 is perhaps its behaviour in methanolic sulphuric acid solution (methanol:5 N-H_2SO_4::4:1). After five days at room temperature the recovered material showed on column chromatographic analysis, besides unchanged KC-6, another Kober chromogen occurring in the oestrone fraction. The ratio of the oestrone-like compound to KC-6 was 2:1. It has been observed that alcoholic solutions of 16-oxooestradiol-17β on prolonged standing at 0° also contained an oestrone-like compound and that this could be quantitatively reverted to 16-oxo-oestradiol-17β with aqueous acid (Section II). Accordingly, the residual mixture was treated in 0.2 N-HCl solution at room temperature for 24 hr. The conversion, however, was not complete, the ratio/

ratio observed being 1:2. It was also found that in contrast to KC-6 both 16α-hydroxyoestrone and 16-oxooestradiol-17β were not converted to an oestrone-like compound in acid methanol.

This phenomenon was also encountered on hydrolysing the KC-6 acetate in acid methanol. The ratio of the 'oestrone component' to KC-6 was 2:1. On further aqueous acid treatment (4 days) the ratio was altered to 1:5.

Acetylation of KC-6

An attempt to isolate KC-6 as its acetate was made when a KC-6 fraction weighing 30.6 mg. and containing 4.4 mg. of Kober chromogen was available. After acetylation with acetic anhydride in pyridine no homogeneous crystalline product could be obtained despite fractional crystallization and alumina chromatography. It was therefore decided to carry out a parallel series of experiments similar to those described earlier, the results of which are mentioned above.

In the Kober reaction the acetate gave as much colour as an equivalent amount of free KC-6. It/

It was not completely resolved from 16α-hydroxyoestrone diacetate in the solvent system 90% ethanol in water:hexane.

Summary of results

The information obtained so far did not favour or disprove a 16, 17-ketolic grouping in ring D. The fairly high degree of stability of KC-6 in alkali and impure ether stood out in sharp contrast to its great blue tetrazolium reducing capacity, though over-emphasis should not be placed on the latter fact in view of the presence of urinary contaminants. However, the formation of two products on sodium borohydride reduction fitted in with the idea that KC-6 might possessed an α-ketolic group. The results of the David reactions with the reduction products, though not very conclusive, lent some support to this idea.

Moreover, obvious differences existed between catalytic reduction in neutral and alkaline solutions; rearrangement of the ketol in alkaline solution might account for these differences.

Finally/

Finally, the formation of an oestrone-like component on acid methanolic treatment suggested its close structural relationship to 16-oxo-oestradiol-17β; the ready formation and partial reversibility of the oestrone-like compound were perhaps only reflecting subtler structural differences between KC-6 and 16-oxooestradiol-17β.

However, it became evident that KC-6 might not be 16β-hydroxyoestrone. The remaining ketolic structure, namely, 16-oxooestradiol-17α, would seem to account for most of the facts. As discussed in Appendix I, 16-oxooestradiol-17α on reduction may give rise to a 1:1 mixture of 17-*epi*oestriol and oestriol-16β, 17α, the unknown oestriol isomer, which is believed to be as 'polar' as oestriol. The finding with the methylated reduction products is in harmony with this idea, for it might well be that the 3-methyl ether of the unknown oestriol would be more 'polar' than oestriol-3-methyl ether. Again, it appears from molecular model studies that the 17α-hydroxyl group is more conveniently placed than its 17β-epimer for cyclic hemiacetal formation with the C-16/

C-16 oxo group. This would then account for the ready formation of the oestrone-like compound in acid methanol and further explain the resistance of KC-6 towards oxidative and reductive (catalytic) processes. However, one formidable objection to this structure is the absence of oestradiol-17α in human pregnancy urine.

Copper acetate oxidation of KC-6

An opportunity to test the α-ketolic structure arose when a paper chromatographic technique for separating oestrone, 16-oxooestrone and 16-oxooestradiol-17β was developed by Marrian & Holdsworth (unpublished work). Huffman (1947) used copper acetate to oxidize 16-oxooestradiol-17β-3-methyl ether to 16-oxooestrone-3-methyl ether. The cupric oxidation of sugars, e.g. ketoses, is of course well known. Thus, if KC-6 were either 16β-hydroxyoestrone or 16-oxo-oestradiol-17α, cupric acetate oxidation should give rise to 16-oxooestrone. The product would show up on the papergram on spraying with the Folin-Ciocalteu reagent (cf. Mitchell & Davies, 1954)/

1954) if the phenolic group remains unaffected.

Some preliminary experiments were carried out with 16α-hydroxyoestrone and 16-oxooestradiol-17β. These results together with those obtained with KC-6 are summarized in the following table.

Table 1,IV.

Results of cupric acetate oxidation experiments with 16α-hydroxyoestrone, 16-oxooestradiol-17β and KC-6.

Compound	Time of reaction (hr.)	Substances detected on papergrams
16α-hydroxy-oestrone	1	16α-hydroxyoestrone, 16-oxooestrone
"	2	16α-hydroxyoestrone 16-oxooestrone, and trace at origin
16-oxooestra-diol-17β	2	16-oxooestrone
KC-6	2	Mainly unchanged KC-6, a weak spot just ahead of 16-oxooestrone, and a spot resembling oestrone
"	2	Mainly unchanged KC-6, and a weak spot just ahead of 16-oxooestrone
"	24	Mainly unchanged KC-6, a weak spot resembling 16-oxooestrone and a fairly strong oestrone spot

Manganese dioxide oxidation of KC-6

Another oxidizing agent was also tried. Manganese dioxide was first employed by Ball, Goodwin & Morton (1948) to convert vitamin A into its aldehyde. Since then Sondheimer & Rosenkrantz (1953) have applied it to the oxidation of steroidal allyllic alcohols. It has not been tried on α-ketols. It seemed of interest to see whether the oestrogenic ketols are oxidized or not. More recently, Barakat, Abdel-Wahab & El-Sadr (1956) have studied the action of MnO_2 on a wide series of organic compounds and thereby shown that this reagent is rather non-specific in its action. Thus, benzoin was found to be readily oxidized to benzil and o-hydroxybenzaldehyde to o-hydroxybenzoic acid.

By paper chromatographic analysis 16-oxo-oestradiol-17β was found to be oxidized to the extent of 90% to two products, one being a bit less'polar'than oestriol and the other remaining at the origin. 16-Oxooestrone when treated similarly, gave rise to two spots identical to those obtained from 16-oxooestradiol-17β. On oxidation/

oxidation with MnO_2 KC-6 yielded, besides unreacted material, one spot remaining at the origin and only a trace of a 16-oxooestrone-like compound.

These oxidation experiments thus rendered improbable the presence of any α-ketolic grouping in KC-6, when taken in conjunction with other previous observations. It seemed to us therefore that concentrates of KC-6 might possibly contain two or more Kober chromogens, the reduction products of which could be readily separated by chromatographic means. This suspicion proved correct with the eventual isolation of two crystalline substances from the crude concentrate.

Isolation of crystalline KC-6A

By leaching a crude KC-6 concentrate obtained from about 250 l. of urine with small volumes of cold acetone, 3.5 mg. of slightly coloured crystals was obtained. The crystalline material was found to contain about 22% of Kober chromogen calculated as oestriol. Its absorption spectrum in concentrated H_2SO_4 after two/

two hours at 25° showed only one absorption peak at about 300 mμ. Visually there was little fluorescence. This was rather surprising since most of the known oestrogens produce intense fluorescence in H_2SO_4 and their absorption spectra in H_2SO_4 have another peak at either 420 mμ or 450 mμ. It appeared that the crystalline material might not be KC-6 itself, but some non-Kober chromogenic material contaminated with a small amount of the Kober chromogen. A study of some of the reactions shown by the KC-6 concentrate with this crystalline substance might help to exclude this possibility. It was crystallized twice from methanol-benzene before use. For convenience, the resultant product was designated acetone-insoluble material (I).

Evidence on the presence of at least two Kober chromogens in KC-6.

At the same time another batch of KC-6 containing 5.75 mg. of Kober chromogen was available. On acetone treatment, the oil, 25.8 mg., afforded 3.7 mg. of crystals (II). A portion of the oil before leaching with acetone/

acetone and the crystalline material were also used in similar studies proposed for the acetone insoluble material (I). Table 2,IV. summarizes the results of these experiments.

Table 2,IV.

Substances tested	Kober colours in O.D. units		Blue tetrazolium O.D. at 510 mμ.
	Before $NaBH_4$ reduction	After $NaBH_4$ reduction	
Acetone-insoluble material (I)	.040 (16)	.100 (38.5)	.006
KC-6 fraction (II)	.300 (22)	.406 (30)	.120 for 23.5μg. of Kober chromogen (i.e. 104 μg. of total fraction)
Acetone-insoluble material (II)	.081 (32)	.140 (51)	.015 for 25μg. solid .061 for 25μg. Kober chromogen

The values given in brackets give the amount of Kober chromogen calculated as oestriol present in per cent.

It will be noted that in each case there was an increase in Kober colour after reduction with sodium borohydride. However, these increases were/

were not proportional to one another, as might be expected, if only one Kober chromogen were present in the KC-6 fraction. The conclusion is inescapable that there were at least two Kober chromogens in the fraction, one of which was a weak Kober chromogen (KC-6A) which on reduction possessed enhanced Kober chromogenicity. The other (KC-6B) seemed to be a strong Kober chromogen, i.e. as chromogenic as an equal quantity of oestriol. It is evident too that the acetone-insoluble material (I) had no blue tetrazolium reducing power. The BT reducing capacities found in both the crude fraction (II) and the acetone-insoluble material (II) might not be due to KC-6B at all.

Assuming that the reduction product of KC-6B differed little from its progenitor in Kober chromogenicity - an assumption now known to be not quite correct, as the reduction product actually gives about 40% less colour than KC-6B - one could obtain a rough estimate of the amounts of the two Kober chromogens present in the crude fraction by means of the data shown in Table 2,IV and proceeding as follows.

Let/

Let x (in O.D. units) be the amount of KC-6B present in the crude fraction,

then we have $0.3 - x$ of KC-6A.

After reduction, the amounts of the reduction products of KC-6A and KC-6B are $0.4 - x$ and x respectively.

Since $\frac{0.3 - x}{0.4 - x} = \frac{.040}{.100}$, we have $x = .233$

Thus, 78% of the Kober chromogens are KC-6B, i.e., $5.75 \times 0.78 = 4.48$ mg.

Since KC-6A is only 16% Kober chromogenic, we have actually $5.75 \times 0.22 \times \frac{100}{16} = 7.9$ mg.

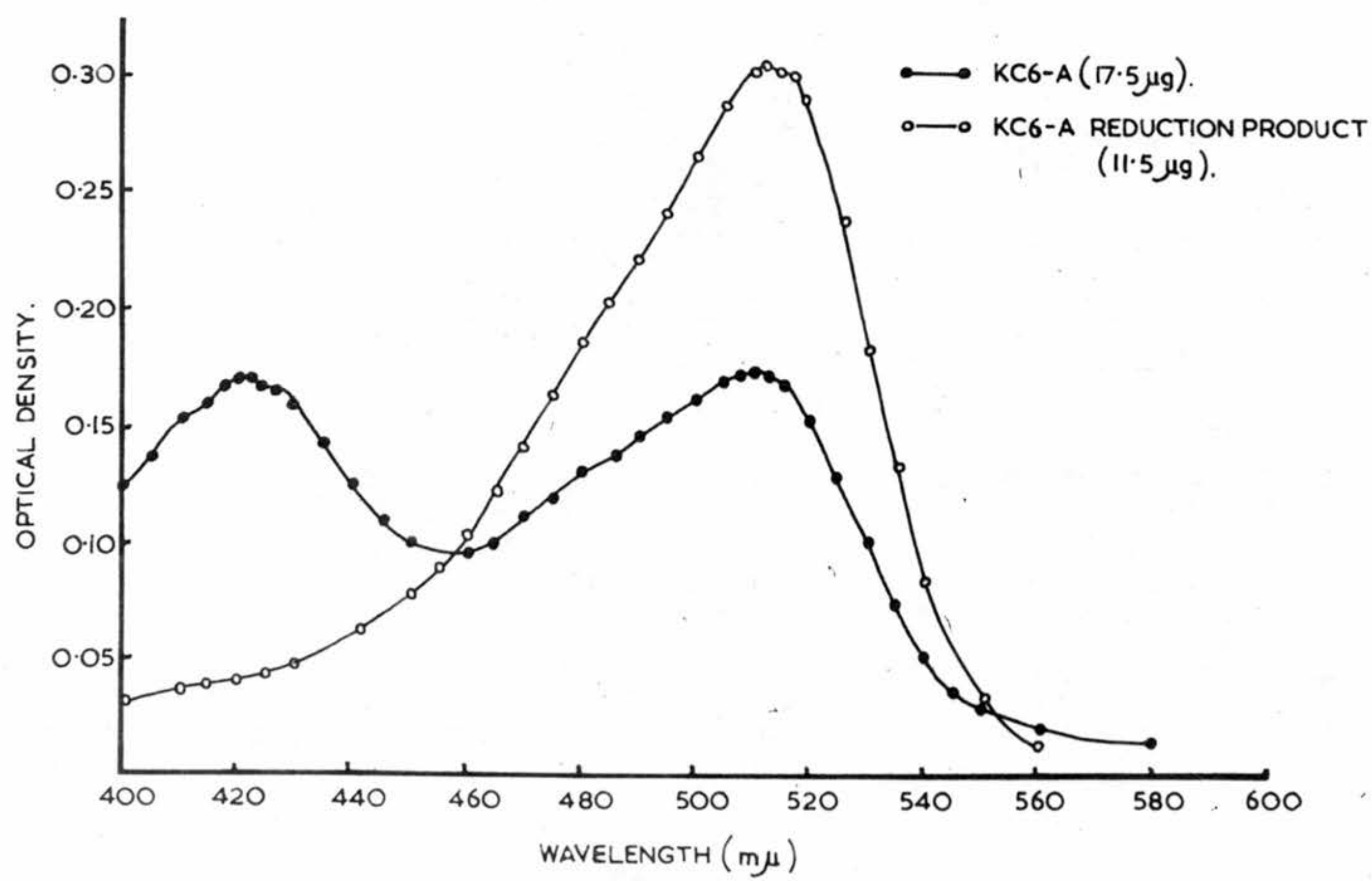
Hence in the total fraction of 25.8 mg., nearly half (by weight) is Kober chromogenic material.

Further purification of crystalline KC-6A

It was subsequently found that leaching with chloroform removed the pigments just as readily as acetone without much loss of KC-6A. Accordingly, the crystalline material recovered from the mother liquors of the methanol-benzene crystallization was combined with the acetone-insoluble material (II). This was leached twice with chloroform and recrystallized once from/

FIG. I, IV.

ABSORPTION SPECTRA OF KOBER COLOURS OF KC6-A AND ITS REDUCTION PRODUCT.



from methanol-benzene to yield 6 mg. of rod-shaped crystals, m.p. 247-250° (evac. sealed tube). It contained 19% Kober chromogen and was negative in the BT and the Zimmermann reactions. On reduction with sodium borohydride there was nearly 200% increase in colour. The reduction product when chromatographed on paper in the system benzene, 70: chloroform, 40: methanol, 75: water, 35, had the same R_f as KC-6A. Thus, the other reduction product resembling oestriol must have been derived from KC-6B.

The absorption spectra of the Kober colours of KC-6A and its reduction product are noteworthy (see Fig. 1,IV); KC-6A has in addition to the maximum at 510 mμ. another maximum of nearly equal intensity at 420 mμ. Furthermore, spectacular differences exist between KC-6A and its reduction product in concentrated H_2SO_4 solutions. The latter is highly fluorogenic and develops a strong yellowish-green colour rapidly.

Infrared/

Infrared spectrum of KC-6A.

The infrared spectrum of KC-6A in a KCl disc was kindly determined by Dr R.K. Callow of the National Institute for Medical Research, Mill Hill, London.

In the OH stretching region there is a single strong band at about 3400 $cm.^{-1}$ with a rather flat maximum. This is rather surprising since the chromatographic property of KC-6A points unequivocally to the presence of at least one alcoholic hydroxyl group. It is possible that the phenolic hydroxyl and the alcoholic hydroxyl absorption bands are not resolved in this instance. On the other hand, Furchgott, Rosenkrantz & Shorr (1946) have shown that in most instances oestrogens give clear spectral differences of alcoholic and phenolic hydroxyl groups. Moreover, by comparing the spectra of oestradiol-17β and its 3-monobenzoate these authors have concluded that phenolic hydroxyl groups tend to absorb at lower frequencies than alcoholic hydroxyl groups. It is well known that/

that associated hydroxyl groups give rise to another absorption band at a lower frequency. Turner (1953) has postulated on the grounds of X-ray and infrared evidence that the phenolic group of crystalline oestrone is intermolecularly associated with the C-17 carbonyl. Jones, Humphries, Herling & Dobriner (1952) have observed that the 17α-hydroxy-20-carbomethoxy groups (bisnor cholanic acid methyl ester side chain) give rise to two bands in the hydroxyl region. The free hydroxyl absorbs near 3620 cm.$^{-1}$ and the associated one near 3500 cm.$^{-1}$ (CCl_4). That the association is an intramolecular one is shown by the fact that a threefold dilution did not influence the intensities of the doublet. Thus, it is possible that the alcoholic hydroxyl in KC-6A is associated probably with the carbonyl group and hence its absorption band merges into the phenolic hydroxyl absorption band.

In the carbonyl stretching region a band with a flattened peak occurs at 1725 cm.$^{-1}$. Information available in the literature on the infrared absorption of a pentacyclic ketone is mainly/

mainly derived from studies in organic solvents, e.g. CCl_4 and CS_2. Thus, a pentacyclic ketone in carbon tetrachloride solution exhibits maximum absorption at 1745 $cm.^{-1}$. It is, however, well established that spectral frequencies in the solid state differ at times considerably from those determined in solution; they are displaced towards longer wavelengths. Hence the 1725 $cm.^{-1}$ band may be ascribed to the C-17 ketone. Meyer (1955) has also observed that steroids with a pentacyclic ketone absorb at about 1725 $cm.^{-1}$ in KBr discs. It may be added, however, that the aldehydic carbonyl absorbs near this frequency. The flatness of the maximum suggests interaction with probably a hydroxyl group.

The bands at ~~1475~~, 1505 and 1610 $cm.^{-1}$ are characteristic of the C=C stretching vibration in an aromatic ring.

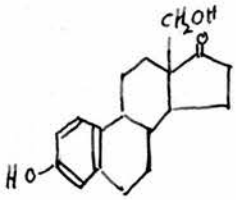
Jones and co-workers (1952a,b) have been able to assign certain group vibration frequencies in the region 1500-1350 $cm.^{-1}$ in steroid spectra to different types of methylene and methyl groups. A band of medium intensity at 1408 $cm.^{-1}$ is/

is associated with a 17-ketosteroid with a free methylene group at C-16. Such a band exists in the spectrum of KC-6A and thus furnishes further evidence to the presence of a C-17 ketone. A medium band at 1377 cm.$^{-1}$ is considered to be characteristic of a C-18 methyl group. Such a band is absent in KC-6A. However, it must be emphasized that the absence of a band at 1377 cm.$^{-1}$ (7.26 μ.) does not necessarily mean that the C-18 methyl group is absent or oxygenated. For example, Furchgott, Rosenkrantz & Shorr (1946) have reported that while oestrone, oestradiol-17β and oestradiol17α-3-monobenzoate have a band at 7.23μ attributableto the C-18 methyl group, no such bands are observed in the case of oestradiol-17β-3-monobenzoate, oestradiol-17α, oestriol, equilin and equilenin. Only bands at 7.17, 7.28, 7.17 (shoulder), 7.32 and 7.18 μ.are present in the spectra of these compounds respectively. More recently, Dr Callow (personal communication) has found that there is no band at 1377 cm.$^{-1}$ in the infrared spectra (KCl) of 16-epioestriol and 16-oxooestradiol-17β. With these reservations in mind one might, however/

however, assume that the C-18 methyl group is oxygenated.

Probable structure of KC-6A.

Three possible structures could be formulated for KC-6A on the basis of the infrared data:



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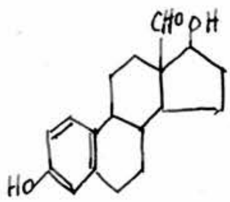
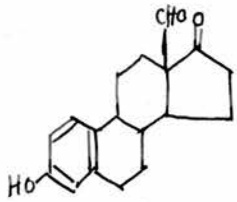


II



III

However, when considered in conjunction with the available chemical and physical information on KC-6A, III could be eliminated. For the reduction product of III would be considerably more 'polar' than III, in actual fact both KC-6 and its reduction product have similar R_f's. Exclusion of either I or II on the basis of their probable chromatographic behaviour is not possible, since interaction effects between the two functional groups would smooth out whatever small differences they may have. The absorption band at 1723 cm.$^{-1}$ does not permit a clear-cut differentiation between/

between I and II. However, the 1408 cm.$^{-1}$ band is absent from the 17β-hydroxy steroids but present in the 17-ketosteroids. Furthermore, the strong dextrorotation of KC-6A ($[\alpha] > +126°$ (ethanol)) certainly favours the presence of a C-17 ketone.

Barton & de Mayo (1954) have shown that methyl hederagonate and icterogenin on treatment with alkali lose formaldehyde; partial cleavage of formaldehyde from the β-ketol group also occurs in strongly acid solution . It seemed that the low Kober chromogenicity of KC-6A might be due to this cleavage of formaldehyde with the formation of an 18-noroestrone, which presumably failed to give the Kober colour. The consistent finding that KC-6 fractions underwent partial destruction on exposure to alkali could be accounted for if KC-6A were 18-hydroxyoestrone.

Mention may also be made on the failure of KC-6A to give the Zimmermann reaction, which has been shown to be specific for 17-ketosteroids (cf. Callow, Callow & Emmens, 1938; also Broadbent & Klyne, 1954). However, in all the 17-ketosteroids investigated so far the C-18 angular methyl/

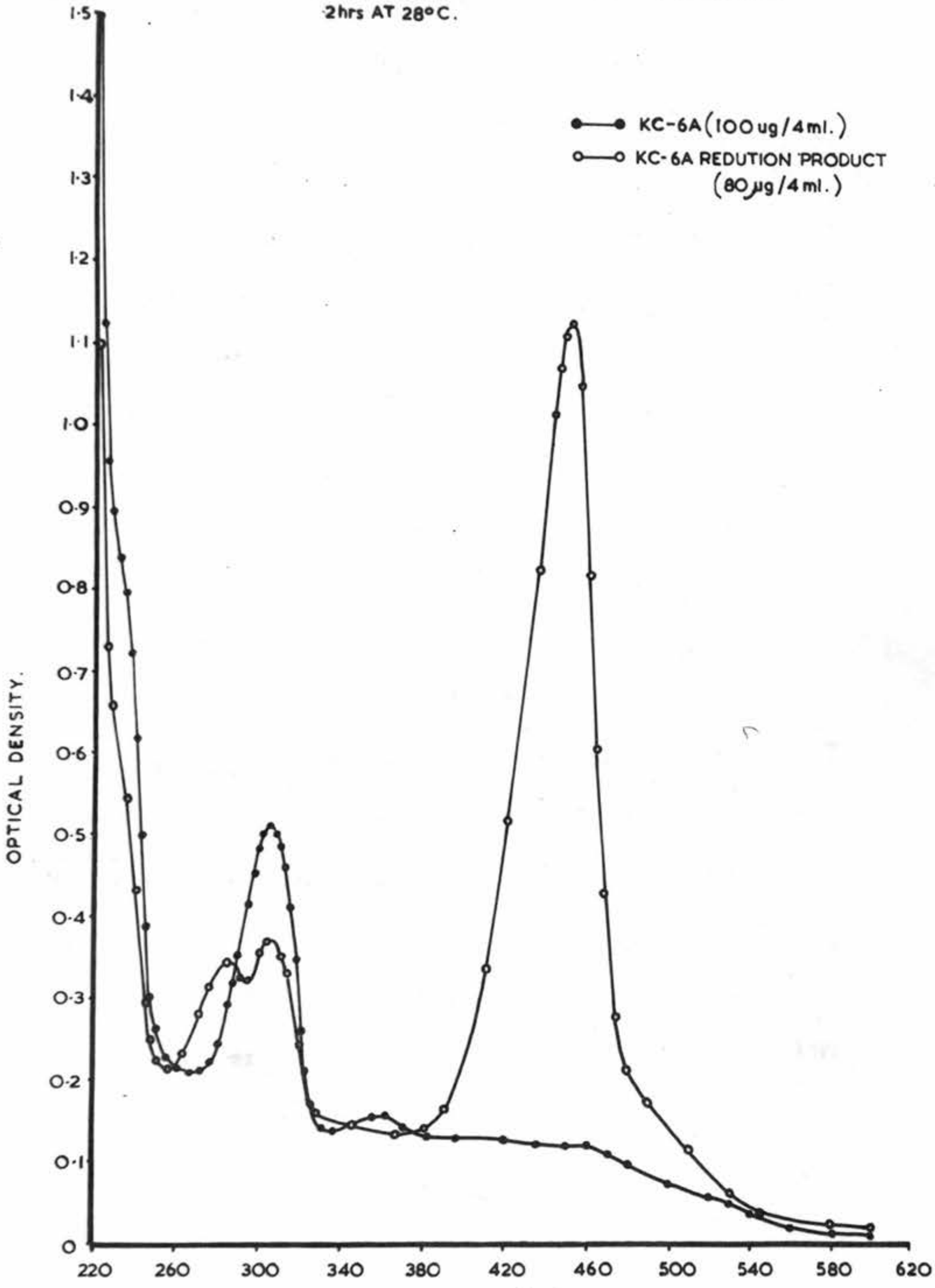
methyl group is present. The reactivity of the α-methylene group at C-16 might have been altered in the event of oxygenation in the angular methyl group or in its absence. It is very likely that loss of formaldehyde takes place rapidly when KC-6A is dissolved in alcoholic KOH, and if this were so, the failure to react must be attributed to the 18-noroestrone formed. The latter compound has a cyclopentanone ring fused to a hexacyclic ring, and, in this connection it is interesting to recall that in the Zimmermann reaction cyclopentanone gave a colour within 5 minutes which faded off at the end of 60 minutes (Callow _et_ _al_., 1938).

Thus, all the available information on KC-6A points to the 18-hydroxyoestrone structure. A ready proof for the assignment of this structure to KC-6A has also emerged from the preceding studies. By degrading KC-6A in alkali the hypothetical products, namely, 18-noroestrone and formaldehyde, might be identified. It might be expected that 18-noroestrone would be similar to oestrone in chromatographic behaviour, but different from oestrone in the Kober reaction. Formaldehyde/

FIG. 2, IV.

H_2SO_4 CHROMOGENS OF KC-6A AND KC-6A REDUCTION PRODUCT.

2hrs AT 28°C.



Formaldehyde might be readily estimated by means of the chromotropic acid reaction. This micro test would suffice for a preliminary identification of KC-6A as 18-hydroxyoestrone.

Preliminary identification of KC-6A as 18-hydroxyoestrone

Using the methods described previously, 11.8 mg. of KC-6A was obtained from 504 litres of urine. This was combined with 4 mg. of material remaining from the previous study. After two recrystallizations from ethanol, analytically pure KC-6A was obtained. Elemental analysis yielded results in close agreement with an empirical formula of $C_{18}H_{22}O_3$. The product had m.p. 255-257° and $[\alpha]_D$ + 146° (ethanol).

Reduction with sodium borohydride yielded a product with similar R_f as that of KC-6A. The H_2SO_4 spectra of these two substances are given in Fig. 2,IV.

The results obtained previously in the blue tetrazolium and the Zimmermann reactions were confirmed.

The/

The acetate of KC-6A was prepared in the usual manner and C and H analyses indicated that both the hydroxyl groups were acetylated. This was confirmed by the absence of free hydroxyl absorption in the infrared spectrum (KCl) of the acetate. In the carbonyl region the 17-ketone and the acetate carbonyl bands were not resolved but appeared as a single band at 1730 $cm.^{-1}$. Two bands at 1208 $cm.^{-1}$ and 1240 $cm.^{-1}$ which are attributable to a phenolic acetate and an alcoholic acetate respectively were present.

The data thus confirmed that KC-6A is an oestrone derivative with an alcoholic hydroxyl group which may be located at C-18. After treatment of KC-6A with N-NaOH at room temperature for 4 hr., 0.90 molar equivalents of formaldehyde (determined by the chromotropic acid reaction) were evolved on acidification and distillation. A ketonic phenolic product was also obtained which gave no colour in the Kober reaction, but was indistinguishable from oestrone when chromatographed on paper in the system benzene, 50: hexane,50: Methanol, 70: water, 30. Thus, it was provisionally concluded that KC-6A is 18-hydroxyoestrone.

Conclusive/

<u>Conclusive proof of the 18-hydroxyoestrone structure for KC-6A.</u>

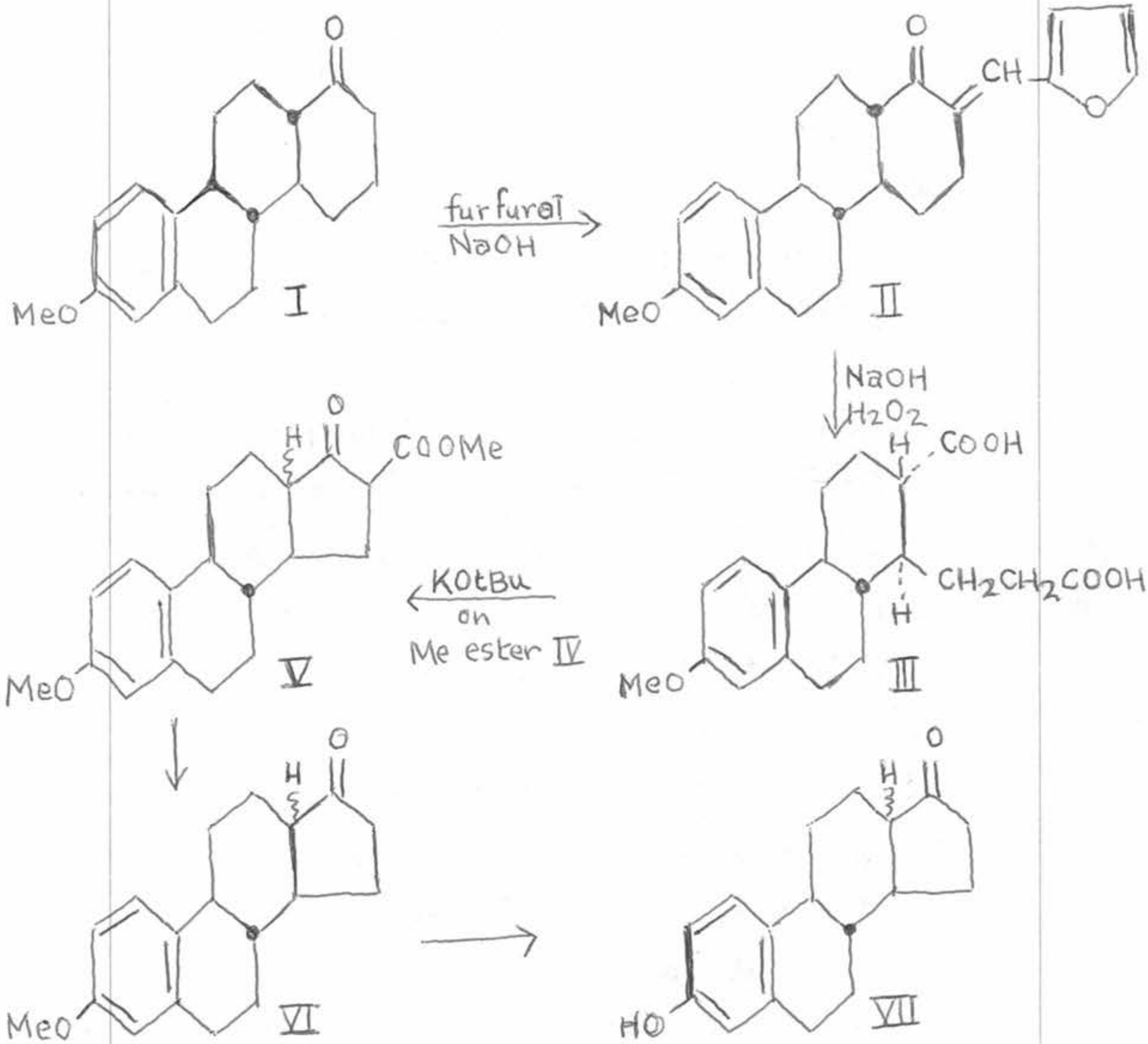
A complete proof of this structure required the isolation of 18-noroestrone and its comparison with authentic material. It was also desirable to identify the volatile component as formaldehyde by its isolation in the form of one of its derivatives, e.g., 2:4-dinitrophenylhydrazone or the dimedone derivative.

The one and only 18-noroestrone described in the literature is that prepared by Robinson & Rydon (1939), who concluded that it had the cis-cis configuration. In the present study the rupture of the bond between C-13 and C-18 might disturb the stereochemistry around C-13 and hence lead to the formation of two racemic mixtures, one having the C/D ring fusion trans and the other C/D cis. However, it is not expected that the B/C ring junction (trans) would be affected in the course of the alkaline degradation. Thus, the 18-noroestrone of Robinson & Rydon would not be identical to either one of the two racemates obtained in this study.

Collaboration/

Chart 1. Total synthesis of dl-18-noroestrone 3-methyl ether

(Johnson, Meyer and Cameron, 1958)



Collaboration was kindly extended to us by Professor W.S.Johnson of the University of Wisconsin. He and his co-workers set out to prepare 18-noroestrone-3-methyl ether by total synthesis as shown schematically on the opposite page.

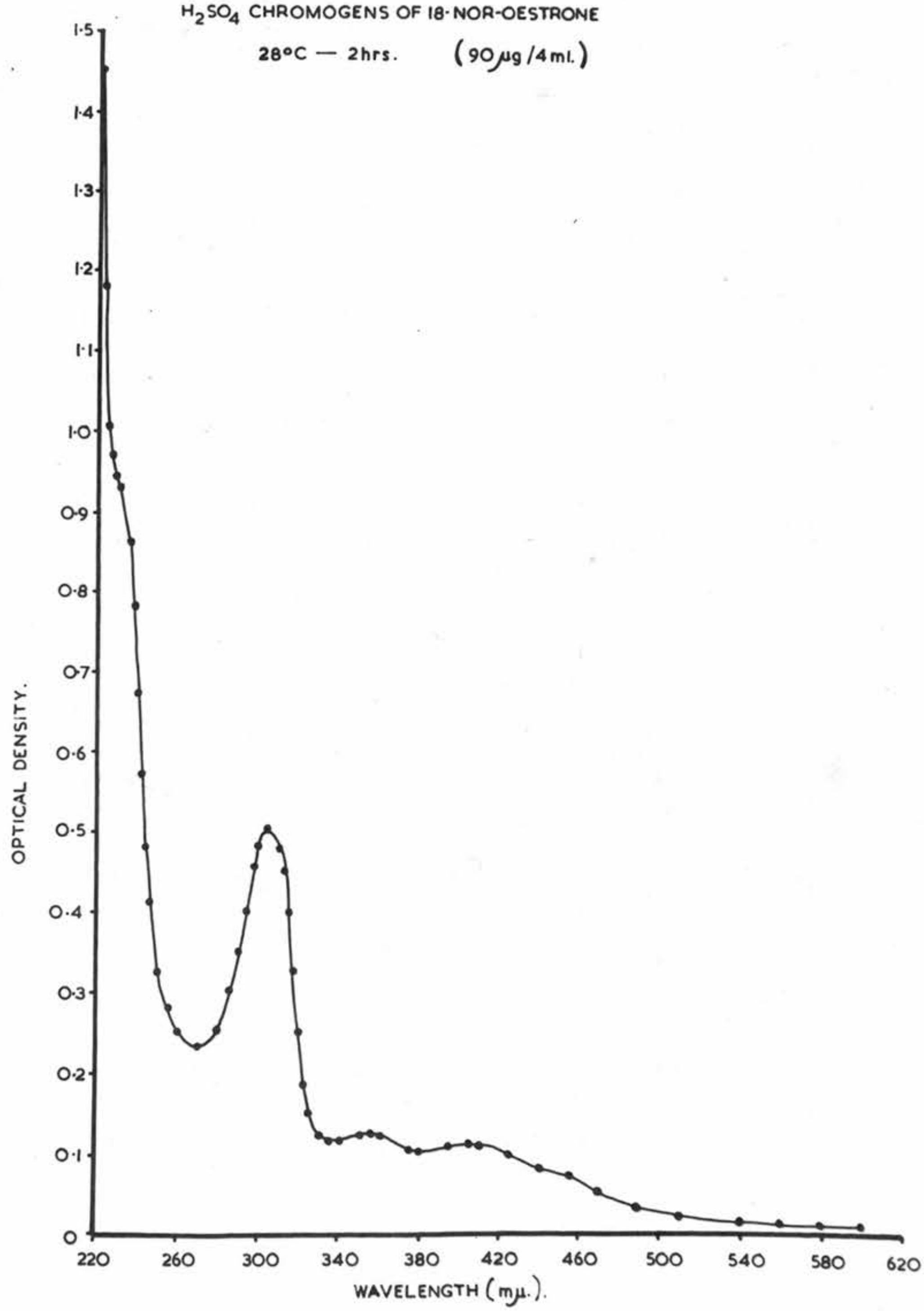
Mention may be made that they have demethylated VI and isolated an 18-noroestrone which on mixed melting point comparison proved to be identical with the material isolated here in a preliminary experiment.

However, since either of the 18-noroestrones obtained is sparingly soluble even in methanol, let alone in carbon disulphide or carbon tetrachloride, it was decided to prepare the 3-methyl ethers for infrared spectrum comparison in solution.

Briefly, by treating KC-6A with N-NaOH at room temperature for 5 hours, liberation of formaldehyde was complete as judged by the chromotropic acid reaction. After acidification of the reaction mixture and extraction with chloroform crude 18-noroestrone was obtained as a/

FIG. 3, IV.

H_2SO_4 CHROMOGENS OF 18-NOR-OESTRONE



a crystalline solid in a yield of 10 mg. Distillation of the aqueous phase into Brady's reagent yielded a product which after purification by chromatography on alumina and crystallization from ethanol did not depress the melting point of authentic formaldehyde 2:4-dinitrophenyl hydrazone.

18-Noroestrone was obtained in fairly pure state after one crystallization from methanol of the crude product. It did not give the Kober or Zimmermann reaction. The absorption spectrum in concentrated H_2SO_4 is noteworthy and very interesting (Fig. 3,IV). The curve is nearly superimposable on that of 18-hydroxyoestrone (Fig. 2,IV) over a wide range of wavelength, thus lending support to the idea that 18-hydroxyoestrone is converted to 18-noroestrone in strongly acid solution.

From the material recovered from the mother liquors was obtained 18-noroestrone 3-methyl ether after treatment with dimethyl sulphate in alkaline solution and purification of the crude product/

peoduct by alumina chromatography and crystallization from methanol. The purified material did not depress the melting point of the higher melting isomer of 18-noroestrone 3-methyl ether prepared by Johnson, Meyer & Cameron. Complete identity of their infrared spectral curves in chloroform solution was demonstrated by Professor Johnson who kindly carried out the comparison. Hence it has been shown that KC-6A is indeed correctly formulated as 18-hydroxyoestrone (Loke, Marrian, Johnson, Meyer & Cameron, 1958).

EXPERIMENTAL

Methods

(a) Paper chromatography

Whatman No. 42 chromatography paper, $4\frac{1}{2}$" x $18\frac{1}{2}$", was washed by continuous extraction with methanol-chloroform (1:1 by volume) in a Soxhlet apparatus for 24 hr. and dried well before use. With unwashed paper longer times were required for elution. A serious objection against using unwashed paper is that with partition solvent systems a band of impurities, which react with the Folin-Ciocalteu reagent, travels near to the solvent front.

With the Bush type of partition systems the chromatographic chamber was kept well equilibrated with the vapour of the two phases in order to get satisfactory results. The spotted paper was allowed to equilibrate for 2-17 hr. before elution with the mobile phase. After development the paper was allowed to dry in air and then sprayed with the requisite reagent.

With the Zaffaroni type of systems, viz., chloroform-formamide, etc., the washed paper was impregnated with a mixture of two parts of methanol and one part of formamide. The excess liquid was removed by blotting with fresh paper. Adhering methanol was allowed to evaporate away at 37° in 45 min. The material to be chromatographed was applied to the paper by means of a blood pipette in a minimum volume of methanol. The paper was then placed in the chromatographic chamber, the bottom of which was covered with mobile phase saturated with formamide. After about 1 hr. development was begun. Running times varied from 4-24 hr., depending on the compounds chromatographed and the solvent systems used. With the Folin-Ciocalteu reagent the paper required no thorough drying before spraying.

All chromatograms were run in a room with its temperature thermostatically controlled at 18°.

(b) Sodium borohydride reduction

To a solution of 100-200 μg. of steroid in 1 ml. of methanol was added *ca.* 5 mg. of $NaBH_4$. After several hours at room temperature the reaction mixture was diluted with 5 ml. 0.2 N-HCl and extracted once with 80 ml. of ether. The ether phase was washed thrice with 5 ml. H_2O and evaporated to dryness. Reductions were invariably quantitative, as was confirmed by the complete absence of ketonic material after Girard separation.

(c) Preparation of micro quantities of oestrogen 3-methyl ethers

The procedure of Marrian & Watson (1956) was used.

To ca. 100 μg. of oestrogen in N-NaOH solution at 37° was added dimethyl sulphate. The reaction vessel was stoppered and shaken vigorously. At the end of 30 min. when the solution was homogeneous further quantities of NaOH and dimethyl sulphate were added and the reaction allowed to proceed for another 45 min. The solution was kept alkaline to litmus throughout. The methyl ether was extracted with ether and the ether extract was washed well with water and evaporated to dryness.

(d) Copper acetate oxidation

A solution of the test material (ca. 100 μg.) in methanol was refluxed in the presence of a few crystals of copper acetate. After cooling, the mixture was diluted with ether and water (weakly acidified) and the ether phase separated, washed twice with water and evaporated to dryness. The residue was then spotted on paper and chromatographed in the system 50% methanol in water:50% hexane in benzene, together with reference compounds.

(e) Manganese dioxide oxidation

A solution of the test material (ca. 100 µg.) in a 9:1 mixture of chloroform and benzene was shaken at room temperature for 3 hr. with excess MnO_2. After filtration the solution and chloroform washings were evaporated to dryness and the residue analysed as described above.

(f) David reaction

To 20-40 µg. of oestrogen in a clean test tube was added 0.6 ml. of concentrated H_2SO_4 and the mixture heated for 10 min. in a boiling water bath. To the cooled mixture were added two drops of arsenic acid, followed by 3 ml. of water. After mixing well, the solution was heated again for 5 min. The blue colour obtained was measured against a reagent blank at its absorption maximum wavelength in the Unicam SP600 spectrophotometer.

(g) Blue tetrazolium reaction

The method of Mader & Buck (1952) has been used. To the compound contained in a glass stoppered test tube were added 0.2 ml. of 95% (v/v) ethanol, 0.04 ml. of 5% (v/v) tetramethyl ammonium hydroxide in 95% (v/v) ethanol and 0.3 ml. of a solution of blue tetrazolium containing 0.10 g. of the tetrazolium salt in 12 ml. of 95% (v/v) ethanol. The mixture was kept in the dark in a water bath at 25°C for 1 hr. The reaction was terminated by the addition of 0.04 ml. of 10% (v/v) acetic acid in 50% (v/v) ethanol and 10 ml. of 95% (v/v) ethanol. The optical density of the test solution was measured against a reagent blank at 510 mμ.

(h) Zimmermann reaction

The modified procedure of Callow, Callow & Emmens (1938) was followed. The oestrogen (20-50 μg.) was treated with 0.2 ml. of ethanol, 0.2 ml. of ethanolic KOH (2.5 N in absolute alcohol) and 0.2 ml. of a 2% (w/v) solution of m-dinitrobenzene in ethanol. After this mixture had been incubated in the dark at 25°C for 60 min., 4 ml. of ethanol were added and the solution finally read against a reagent blank at 440, 520 and 600 mμ. The Allen correction formula was applied to provide the corrected optical density at 520 mμ.

(i) Melting point determinations

All melting points, unless stated otherwise, were determined on a hot-stage microscope apparatus of the Kofler type and are uncorrected.

Isolation of crude KC-6

Enzyme hydrolysis of urine

Urine specimens were acidified to pH 4.7 with acetic acid, buffered to this pH by the addition of one-tenth of their volume of M-acetate buffer, and incubated at 37° with the enzymic preparation from Patella vulgata (1,000,000 units Fishman units/l. of urine) for a period of 48 hr. In every instance half of the total amount of enzyme used was added initially and half after 24 hr.

Method of extraction

After enzymic hydrolysis 600 g. of NaCl were dissolved in each 4 l. batch of urine in order to facilitate the subsequent extraction of oestrogens into ether and to minimize stable emulsion formation during ether extraction. The urine was then extracted once with an equal volume of peroxide-free ether. The extract was washed once with one-tenth of its volume of 5% (w/v) $NaHCO_3$ and then extracted twice with one-eighth volume of cold N-NaOH. The alkaline extract was partially neutralized by the addition of 5N-H_2SO_4, and finally brought to pH 9.0-9.3 with gaseous CO_2. In more recent work the alkaline extracts were immediately acidified with excess 5N-H_2SO_4 to minimize losses of KC-6A which occur rapidly in strongly alkaline solutions.

The neutralized extract was extracted with equal volumes of ether, the extract washed twice with one-quarter volumes of water, dried over Na_2SO_4 and evaporated to dryness. This was the 'phenolic' fraction.

Girard separation

Phenolic fractions from about 100 l. of urine were combined and treated at room temperature for 17 hr. with 5 g. of trimethylammonium-aceto-hydrazide chloride in 50 ml. of ethanol and 10 ml. of acetic acid. The mixture was then thoroughly chilled and 90% of the acid neutralized by NaOH in 500 ml. of a solution which also contained 50 g. NaCl. The non-ketonic material was then removed by extraction once with four-fifths and thrice with two-fifths of its volume of ether. The remaining aqueous ketonic fraction was acidified with 60 ml. concentrated HCl and allowed to stand at room temperature for 1 hr.

The ketonic phenolic fraction was extracted from the acidified mixture four times with two-fifths of its volume of ether. The combined ether extract was washed three times with one-fifth of its volume of 8.5% (w/v) $NaHCO_3$, twice with one-fifth of its volume of water, dried over Na_2SO_4, and evaporated to dryness.

Partition chromatography

The phenolic ketonic fraction from 100-200 l. of urine was applied by either of the following two methods on to the top of a Celite column (120 g.; 2 cm. diameter). In method (a) the fraction was dissolved in 3 ml. of stationary phase and quantitatively transferred, with the aid of a further 2 ml. of stationary phase, to 5 g. of Celite contained in a small beaker. After thorough mixing of the stationary phase with the Celite, mobile phase was added to prepare a slurry which was transferred and packed on to the top of the column. A total of 40 ml. of mobile phase was used to effect quantitative transfer. In method (b) the ketonic fraction was rendered miscible with 20 ml. of the mobile phase by the prior addition of 0.2 ml. of methanol. This was pipetted onto the column, followed by two further transfers of 10 ml. of mobile phase each. Sixty 40 ml. fractions of eluate were collected automatically. Fractions 1-15, which contained oestrone, etc., were combined, evaporated to dryness under reduced pressure and stored at 0°. Likewise, fractions 16-28 and 29-48, which contained the KC-5 and the KC-6 respectively, were pooled and processed up as described above.

For the isolation of KC-6A a second

partition chromatography was carried out in the same manner to free the fraction of some KC-5.

Isolation of KC-6A (from 504 l. of urine).

Fractions 29-48 from the second chromatogram were combined, yielding 84 mg. of brown oil, which was found to contain 8 mg. of Kober-chromogen. By leaching twice with chloroform at -20°, 12.4 mg. of slightly pigmented solid was obtained. This was crystallized once from methanol-benzene to yield 11.8 mg. of material. After this had been combined with 4 mg. of similarly prepared material from an earlier batch of urine it was leached with chloroform (no loss in weight) and then recrystallized once from methanol-benzene. A colourless product of 11.4 mg. was obtained, m.p. 256-260°(evac. sealed tube). For analysis it was crystallized once more from ethanol at 5°, yielding 5.7 mg. of pure KC-6A. After drying to constant weight at 100° in vacuo the product had m.p. 255-257°, subliming at about 210° (evac. sealed tube); $[\alpha]_D^{19}$ + 146° (C, 0.369 in ethanol); ε 281, 2200 in ethanol. (Found: C, 75.4; H, 7.9. Calc. for $C_{18}H_{22}O_3$: C, 75.5; H, 7.8%) The infrared spectrum (KCl disc) was kindly determined by Dr Callow.

Properties and characterization of KC-6A

In the Kober reaction 10 µg. of KC-6A gave colour equivalent to 2.2 µg. of oestriol. After reduction with Na borohydride the product (ca. 10 µg.) produced colour equivalent to 5.4 µg. of oestriol. The increase in colour was about 133%. On paper chromatography in the system benzene, 70:chloroform, 40:methanol, 75:water, 35, the product appeared as one spot with R_f (about 0.5) similar to those of KC-6A and 17-*epi*oestriol. The $NaBH_4$ reduction was used to check the various purification steps mentioned above.

In the blue tetrazolium reaction KC-6A was negative. It was also negative in the Zimmermann reaction.

Solutions of KC-6A (100 µg.) and its reduction product (80 µg.) in 4 ml. concentrated H_2SO_4 were allowed to remain at 24° for 2 hr. Absorption spectra over the range 220-600 mµ. were determined on both solutions (Fig. 2,IV).

KC-6A diacetate

The material recovered from the optical rotation measurement was combined with 2.3 mg. of KC-6A obtained by recrystallization of the material recovered from the mother liquors. The combined material was acetylated in the usual manner. The product, 7.7 mg., was recrystallized twice from ethanol at 5°, yielding 3 mg. of leafy plates, m.p. 162-165°. For analysis it was dried to constant weight at 100° in vacuo. (Found: C, 70.8; H, 7.0. Calc. for $C_{22}H_{26}O_5$: C, 71.3; H, 7.1%) The infrared spectrum (KCl disc) was carried out on the remaining material.

Micro-scale alkaline degradation of KC-6A

Method (a)

A solution of 100 μg. of KC-6A in 2 ml. N-NaOH in a stoppered distillation flask was set aside at room temperature. A control without steroid was set up. After 4 hr., 3 ml. of 10 N-H_2SO_4 and 4 ml. of water were added to the control flask and distillation was carried out in the micro distillation apparatus (previously flushed with steam). The distillate was trapped in 1 ml. of 4% Na_2SO_3 solution contained in a test tube graduated at 5 ml. and 7 ml. Distillation was discontinued when the volume of the liquid in the tube was 5 ml. and after washing down the tip of the condenser the distillate was diluted to 7 ml. with water. The test was carried out in a similar way. A 2 ml. aliquot was removed from each distillate and reacted with 3 ml. of 0.2% chromotropic acid in 30 $N-H_2SO_4$ at 100° for 30 min. After cooling, the intensity of the colour developed was measured at 570 mμ. against the 'control' and the amount of formaldehyde read off a calibration curve. The yield was 90% (9.45 μg.).

The liquid residue in the distillation flask was diluted with water and ether. The ether phase was separated, washed with $NaHCO_3$ and water, and evaporated to dryness. The residue was dissolved in a small volume of methanol and a one-fifth aliquot spotted on paper. When chromatographed against oestrone in the system 70% methanol in water:benzene-hexane (1:1, by vol.), the product was indistinguishable from oestrone in mobility.

Method (b) (cf. Barton & de Mayo, 1954)

A solution of 50 μg. of KC-6A in 1 ml. of N-NaOH was set aside at room temperature. After 4 hr. it was acidified with 0.6 ml. $5N-H_2SO_4$ and transferred quantitatively to a small separating funnel with 0.4 ml. of water. The aqueous phase was extracted twice with 1 ml. of chloroform, care being taken that complete separation of the two phases occurred before withdrawal. The aqueous phase was transferred to a test tube, 3 ml. of chromotropic acid added, and the mixture heated in a water bath for 30 min. The yield of formaldehyde was 83.8% (4.4 μg.).

The chloroform extracts were combined, diluted with more chloroform and washed with water. The recovered material was separated into non-ketonic and ketonic fraction, and the ketonic residue (one-fifth) was chromatographed with oestrone on paper as described above. Two spots with similar $R_{f's}$ were obtained.

Degradation of KC-6A and isolation of products

By methods similar to those described above a further batch of KC-6A was obtained in a yield of 16 mg. from 400 l. of urine. After three crystallizations from methanol a product was obtained which showed a double melting point (ca. 220° and 248-257°) (evac. sealed tube).

A solution of KC-6A recovered from the mother liquors (11.2 mg.) in 200 ml. N-NaOH was set aside at room temperature. Portions of the solution (0.5 ml.) were withdrawn at various time intervals and the aqueous phase after acidification and chloroform extraction was treated with chromotropic acid reagent as described above (method (b)). Liberation of formaldehyde was essentially complete after 1 hr. At the end of 5 hr. the reaction mixture was acidified with concentrated HCl (17.5 ml.) and, when cool, was extracted three times with one-third volume of chloroform. The combined chloroform extract was washed once with 40 ml. of 5% (w/v) Na_2CO_3 (which was back-washed with 40 ml. of chloroform), and three times with 20 ml. of water. The back-wash, after being washed twice with water, was combined with the main bulk, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue weighed 10 mg.

Identification of volatile product

The aqueous phase after further acidification was distilled into 50 ml. of Brady's reagent until a volume of about 150 ml. was reached. The aqueous phase was extracted twice with 50 ml. of ether/

ether and the combined ether extract was then washed with 10 $N-H_2SO_4$ (6 x 5 ml.) and water (6 x 8 ml.), dried and evaporated to dryness. The product was grossly impure and could not be purified by crystallization from ethanol. It was finally chromatographed on a small alumina column in benzene. The crude product was recovered in the early fractions. After three recrystallizations from ethanol, fine slender needles, m.p. 160-162.5°, were obtained. When mixed with authentic formaldehyde 2:4-dinitro phenylhydrazone (m.p. 162-164), the melting point was 161-163°.

Identification of solid product

The solid product obtained above was leached once with 0.2 ml. methanol to remove pigmented material. The resultant product, 7 mg., was crystallized once from methanol, yielding 1 mg. of rod-shaped crystals. It was homogeneous chromatographically and was indistinguishable from oestrone in two solvent systems. The absorption spectrum of 90 μg. of this product in concentrated H_2SO_4 (4 ml.) was determined from

220-600 mμ. (Fig. 3, IV). It did not give a Kober colour and was negative in the Zimmermann reaction. However, when spotted on paper and sprayed with the Zimmermann reagent, a purplish-red spot was discernable.

The recovered material was combined with 2 mg. of solid product obtained from a previous batch of KC-6A. This (11 mg.) was methylated with dimethyl sulphate (0.3 ml.) in N-NaOH (9 ml.) at 37°. After 60 min. another 3 ml. of 10% NaOH solution and 0.5 ml. of dimethyl sulphate were added. At the end of another 60 min. the aqueous phase was extracted with ether. On removal of solvent, 8.5 mg. of crude methyl ether was obtained. Crystallization from methanol or ethanol did not afford a pure product. For instance, 1.5 mg. of material was obtained after crystallization from ethanol at -20°, m.p. 135-147°, sintering from 120°. The combined material was finally chromatographed on 1 g. of alumina (deactivated with 5% (w/w) of water), prepared in a benzene-hexane (1:2) mixture. Successive 2 ml. fractions were collected and the desired product, 4.9 mg., was recovered in 5 fractions on elution with benzene-hexane. After one crystallization from methanol at -20°, 1.5 mg. of crystals (flat plates) was obtained, m.p. 145-149°. Another crystallization from methanol afforded 1.3 mg. of material, m.p. 143-147°. Mixed with the synthetic methyl ether provided by Professor Johnson (m.p. 145-147°), the melting point was 142-147°. The solution ($CHCl_3$) infrared spectra of these two substances were identical.

4. Discussion

18-Hydroxyoestrone is the fourth steroid with an oxygenated C-18 carbon to be isolated from mammalian sources. The first of these is aldosterone isolated by Simpson and co-workers (1953) and Mattox, Mason & Albert (1953) from ox adrenal glands; the second being 18-hydroxydeoxycorticosterone obtained by incubating DOC with ox adrenal homogenates (Kahnt, Neher & Wettstein, 1955). The third is 18-carboxy-deoxycorticosterone isolated in the hydroxy-lactone form ($18 \longrightarrow 20$) by Neher & Wettstein (1956) from hog and bovine adrenals. 18-Hydroxyoestrone is, however, the first 18-hydroxylated steroid to be isolated/

isolated from human urine.

Incidentally, these three C-18 oxygenated steroids of the C-21 series known at present happen to fall into the following chemical sequence:

primary alcohol $\rightleftarrows$ aldehyde $\rightleftarrows$ carboxylic acid

It will be interesting to establish whether such a metabolic relationship exists in the case of 18-hydroxyoestrone.

In the chemical field C-18 oxygenated steroids have already been reported. The total synthesis of aldosterone has been achieved by two groups of Swiss workers (Schmidlin, Anner, Billeter & Wettstein, 1955; Lardon, Schindler & Reichstein, 1957). Recently, McNiven (1957) prepared 18-aldehydo-pregnane from conessine; the former being then converted to the 18-carboxy and 18-hydroxy derivatives. It was noted that the 18-aldehydic group is rather sensitive to oxidative processes and undergoes partially spontaneous oxidation to the acid.

It is quite likely that 18-hydroxyoestrone might have been formed from 18-aldehydo-oestrone by a/

a Canizzaro type of reaction in the presence of alkali. However, since KC-6A has recently been detected in the ketonic fraction of neutral-phenolic extracts, obtained from pregnancy urine under conditions in which excessive alkaline treatment is avoided, it is safe to conclude that 18-hydroxyoestrone is a true metabolite of oestrone.

The amount of KC-6A in pregnancy urine seems to be fairly high. While yields based on isolation data averaged about 3 mg./100 l. of urine, there is reason to believe that the amount excreted might be 2-3 times this. For one thing not all the KC-6A separated out on leaching the crude fraction with chloroform and an appreciable amount still remained in the KC-6B fraction. Moreover, the fractionation and extraction procedures were by no means quantitative, while the losses occurring during the phenolic separation are now believed to be quite high. Thus, in examining oestrone fractions by paper chromatography in the benzene-formamide system, which separates oestrone from 18-noroestrone, Dr Layne found appreciable quantities of a compound resembling/

resembling 18-noroestrone. A rough estimate placed this at about 10 mg./700 l. of urine.

Considering these together, it seems that the amount of KC-6A excreted in late pregnancy is about a third that of oestrone. It is thus a fairly important metabolite from the quantitative standpoint. Whether it is excreted to any significant extent during the menstrual cycle - the answer to this must await the development of a quantitative method.

As will be seen in a subsequent section, 18-hydroxyoestrone seems to be formed from oestrone in the presence of ox adrenal whole homogenates. Though there may not be a close relationship between the human and the bovine species, it is reasonable to assume that in man oestrone also undergoes 18-hydroxylation in the adrenal cortex. One cannot exclude just now the ability of the placenta or the ovary to effect 18-hydroxylation.

No biological tests have yet been carried out with 18-hydroxyoestrone; hence its biological functions must remain obscure for the time being. In/

In so far as aldosterone with a C-18 oxygen function is a very potent mineralocorticoid, it may be speculated if 18-hydroxyoestrone (or its derivatives) would affect electrolyte metabolism during pregnancy.

Meyer (1955) has demonstrated that 19-hydroxylation is a prerequisite step in the aromatization of ring A of androst-4-ene-3,17-dione. The introduction of a 2-hydroxyl group and subsequent dehydration to give a 1,2-double bond is believed to be another step (cf. Dorfman, 1956). More recently, Kushinsky (1958) isolated besides oestradiol-17β-4-C^{14} 17-monoacetate, a 1- or 2-hydroxy derivative of 19-nortestosterone acetate-4-C^{14}, from the incubation medium of 19-nortestosterone acetate-4-^{14}C with Corynebacterium simplex. It has also been shown recently that androst-4-ene-3,17-dione undergoes 1α- and 2β-hydroxylation on incubation with Penicillium Sp (ATCC12556) and that the 1α-hydroxy derivative is readily aromatized to oestrone on pyrolysis (Dodson & Muir, 1958). Evidence is produced in Section V showing that oestrone undergoes ~~both~~, 18-/

18-, 11β- and, very probably, 12α-hydroxylation on incubation with ox adrenal homogenates. It may well be then that these hydroxylations are but stages in the aromatization of ring C.

Though the means by which aromatization takes place remains to be elucidated, the ability of the mammalian organism to do this is no longer questioned. The recent isolation of equilenin by Salhanick & Berliner (1957) from an adreno-carcinoma tumour of a male - an oestrogen thought to be confined to the equine species - is of particular interest. Whether it is produced <u>de novo</u> or derived from oestrone remains to be seen. In any case, it appears that adequate biological mechanisms exist for the aromatization of rings A, B and probably C with the production of a phen-anthrene derivative. This is germane to the question of carcinogenesis.

Years ago Fieser (1941) postulated that 3-hydroxycholanthrene - a potential carcinogen - might be formed by the condensation of equilenin (or oestrone) and pyruvic acid and subsequent dehydrogenation <u>in vivo</u>. In view of the non-potency/

potency of cholanthrene hydroxylated derivatives in the production of tumours, Fieser was not too sanguine with regard to his hypothesis. The isolation of 3-deoxyequilenin from pregnant mares' urine (Prelog & Fuhrer, 1945) and the more recent demonstration of a dehydroxylation mechanism in the rat and the rabbit (DeEds, Booth & Jones, 1957) suggest that dehydroxylation is just as normal as, if not more common than, hydroxylation. Hence one of the most formidable obstacles that stand in the way of the biological conversion of oestrogen to carcinogens no longer exists.

In view of this a quantitative method for the determination of 18-hydroxyoestrone is considered desirable. Much experimentation has to be done, however, in the evolution of such a method. The two major obstacles are (1) its lability to alkali, and (2) its weak Kober chromogenicity as well as its poor fluorogenic capacity. The former can be offset by avoiding the phenolic separation, while the latter may be surmounted by reducing 18-hydroxyoestrone before chemical assay.

SECTION V. THE IN VITRO METABOLISM OF OESTRONE BY OX ADRENAL CORTICAL HOMOGENATES

Introduction

The isolation of 18-hydroxyoestrone from human pregnancy urine raises the interesting problem of its biosynthesis. While it is probable that it is derived from oestrone by the *in vivo* introduction of oxygen into the C-18 methyl group, the site and the mode of such a hydroxylation require elucidation.

The hydroxylation of the C-19 methyl group of deoxycorticosterone by ox adrenal homogenates has been demonstrated by several groups of workers (Kahnt, Neher & Wettstein, 1955; Hayano & Dorfman, 1955; Zaffaroni, Troncoso & Garcia, 1955). Similarly, Levy & Kushinsky (1955) reported the isolation of small quantities of 19-hydroxy-deoxycorticosterone after perfusion of 90 g. of progesterone through 600 ox adrenals; whether 19-hydroxylation preceded 21-hydroxylation or *vice versa* in this instance, is not known. The in/

in vitro conversion of dehydroepiandrosterone to 19-hydroxy-androst-4-ene-3:17-dione by bovine adrenal homogenates is a classical example of 19-hydroxylation in the C-19 steroids (Meyer, 1955).

However, the only recorded instance of 18-hydroxylation is that of Kahnt, Neher & Wettstein (1955). These authors obtained, among other products, the 18-hydroxy derivative of deoxycorticosterone by the action of ox adrenal homogenates on this steroid.

The work described in this section was therefore undertaken with the view of demonstrating 18-hydroxylation in the oestrogen series by ox adrenal homogenates. Oestrone was used as the substrate and several of its metabolites were obtained and provisionally identified.

Results

Preliminary small-scale experiments.

These experiments carried out as described by Kahnt, Neher & Wettstein (1955) yielded results which are summarized in the following table. For convenience, the terms KC5 and KC6 will be used to indicate the metabolites detected in/

in the chromatographic fractions as obtained in the urinary isolation work, though these metabolites might not necessarily be 16α-hydroxyoestrone (or the other ring D ketols) and 18-hydroxyoestrone respectively.

Table 1,V.

Conversion of oestrone to KC-5 and Kc-6 by ox adrenal homogenates.

Expt. No.	Kober chromogen detected(μg.)		Folin-Ciocalteu chromogen detected (μg.)		Yield of KC-6A(based on HCHO liberated)
	KC-5	KC-6	KC-5	KC-6	
1	trace	trace	-	-	-
2	(a)1.3	-	-	2	
	(b) -	-	-	5	1.2[x]
3	(a)1.2	1	ca 5	ca 5	-
	(b) -	-	-	10	2.5[x]
4	2	-	-	4	-
5	-	-	-	2	-

[x] Oestrone-like spot detected in residue after alkaline treatment

- Not carried out

Each determination except in (b) was carried out on an aliquot containing originally 500 μg. of oestrone.

These results seemed to indicate that there were formed ca 0.2% of the KC-5 metabolite and a similar yield (based on HCHO liberation test) of KC-6A. It will be noted that the formaldehyde liberated in the latter instance was only one-fourth of that expected. However, the presence of an oestrone spot on the paper chromatogram after alkaline treatment provided additional evidence to the provisional conclusion that 18-hydroxyoestrone (i.e. KC-6A) was formed from oestrone by 18-hydroxylation.

Further small-scale experiments

Before embarking on large-scale experiments to obtain sufficient material for adequate characterization of this and other metabolites, some further small-scale experiments were carried out. Unfortunately, the analytical technique was simplified in that the detection and estimation of the metabolites were confined to the use of the Folin-Ciocalteu reaction. Though more sensitive than the Kober reaction as far as weak Kober chromogens are concerned, it is less specific than the latter. In consequence no attempts were made to repeat the other chemical tests as carried out in the preliminary experiments.

(a) Effect of ATP on the enzymic hydroxylation of oestrone

Since some preparations of ATP contain traces of heavy metals, which could inhibit certain enzymic hydroxylations (Grant, personal communication), the effect of ATP requirement in the hydroxylations encountered in this work was investigated. Moreover, if it were subsequently found that ATP is not essential, its deletion from the co-factors to be added to the large-scale incubation mixture would help to keep down the cost. By comparing the yields obtained in the presence and absence of ATP, it was concluded that no significant increase in yield occurred in the presence of ATP under the conditions employed.

(b) Effect of TPN on the enzymic hydroxylation of oestrone

Similarly, the effect of the deletion of TPN, its reduced form being essential for the 11β-hydroxylation of neutral steroids (cf. Grant, 1956; Sweat & Lipscomb, 1955), was also investigated, using a modified incubation medium. The TRIS buffer was adopted because of its greater buffer capacity and its enhancing effect on 11β-hydroxylation (Grant & Brownie, 1955). It was found/

found that yields in the presence of TPN were certainly higher (1-2 times), but in view of the scarcity of this co-factor, it was felt that the difference in yields might be made good by adding more DPN than was used before, i.e., 3 times.

(c) To investigate whether the zona glomerulosa or zona fasculata contains the higher hydroxylating activity

Since Ayres, Hechter, Saba, Simpson & Tait (1957) have demonstrated that capsule strippings of ox adrenals, previously shown to be mainly zona glomerulosa tissue (Ayres, Gould, Simpson & Tait, 1956), facilitated the conversion of progesterone, deoxycorticosterone and corticosterone to aldosterone - one of the metabolic steps presumably involves 18-hydroxylation - the hydroxylating activities in regard to oestrone of the zona glomerulosa and zona fasiculata were investigated. Again, by means of a convenient but by no means quantitative method of estimation, it was found that the zona glomerulosa cells contained greater hydroxylating activity as far as the KC-6 metabolite was concerned. However, when considered from the point of view of large-scale/

scale work, the use of the zona glomerulosa zone alone would require far too much biological material and longer times in working up the enzyme preparations. It was therefore decided to use the whole cortex with a consequent though not unexpected fall in yield (from about 10% to 2-3%).

Large-scale experiments

With the conditions adequately established, several large-scale experiments were carried out. It was, however, found that emulsions, which were also encountered during the alkaline extraction in the small-scale experiments, became rather serious, particularly when TRIS buffer was used. Accordingly, the phosphate buffer of Kahnt *et al.* was employed again. Even then it required just as much Bradosol as before to effect the breaking up of the emulsions. The time was, however, shortened.

From twelve large-scale experiments a crude phenolic fraction of 318 mg. was obtained from roughly 180 mg. of oestrone. After removing as much oestrone as possible by fractional crystallization /

crystallization, 161 mg. of a semicrystalline residue was obtained. This was chromatographed on a Celite column and the various fractions worked up. The oestrone fraction was not investigated.

Fractions 7-12 in which oestradiol-17β and dioxo oestrogens might occur, contained Kober chromogenic material, which appeared entirely subsequently in the non-ketonic fraction. It was therefore concluded that this metabolite (1.6 mg. by weight) was oestradiol-17β which must have arisen from the metabolic reduction of oestrone.

The ketonic fractions of the chromatographic fractions 13-20 contained none of the spots which were previously detected on paper chromatograms of the incubation mixtures, and hence was not further investigated.

Fractions 21-29 yielded material which was further purified by means of the Girard reaction. The ketonic fraction on paper chromatographic analysis in the chloroform - formamide system was shown to contain two of the four metabolites. These were designated x_1 and/

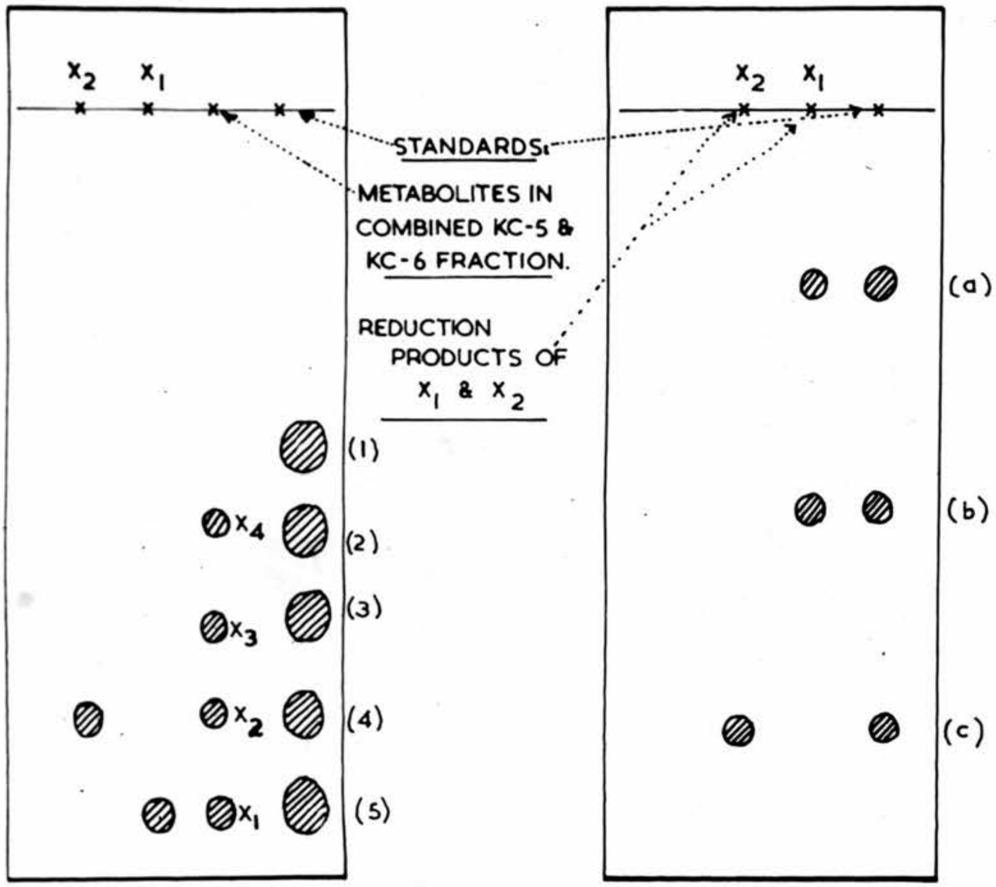
FIG. I, V.

(a) SOLVENT SYSTEM.

CHLOROFORM/FORMAMIDE.

(b) SOLVENT SYSTEM.

CHLOROFORM/FORMAMIDE.



TIME OF DEVELOPMENT, 10-12 hrs.

STANDARDS.

1) 16α-HYDROXYOESTRONE.
2) 16-OXOOESTRADIOL-17β.
3) 16β-HYDROXYOESTRONE.
4) 17-<u>EPI</u>OESTRIOL.
5) 16-<u>EPI</u>OESTRIOL.

TIME OF DEVELOPMENT, 22-24 hrs.

STANDARDS.
? OESTRIOL

a) 16-<u>EPI</u>OESTRIOL. ADIOL
b) 11β-HYDROXYOESTRIOL.
c) OESTRONE. ? 16 epi

and x_2 and were similar to 16α-hydroxyoestrone and 16-oxooestradiol-17β respectively in chromatographic mobility. These were separated on a preparative paper chromatogram and further analysis showed that the separation was complete. The yields as judged by the visual intensities of the F-C spots, were estimated to be about 700 and 500 μg. respectively; while based on the Kober colours, these were 170 and 185 μg. respectively.

Identification of x_1. In the Zimmermann reaction x_1 was very weakly positive. On reduction with sodium borohydride in methanolic solution x_1 yielded two products which had the same R_f's as oestriol (and 6β-hydroxyoestradiol-17β) and 11β-hydroxyoestradiol-17β when chromatographed for 24 hr. in the chloroform-formamide system (Fig. 1, V). The ratio of the yields of these two reduction products was about 6 to 1. Thus, besides 11β-hydroxyoestrone, x_1 might contain 6β-hydroxyoestrone and/or 16α-hydroxyoestrone. Since 16α-hydroxylation has not previously been demonstrated in the adrenal, the/

the view that the major metabolite in x_1 was 6β-hydroxyoestrone was therefore favoured. However, when it was later found that x_2 might consist mainly of 16-oxooestradiol-17β (see below), further experiments were carried out with x_1 to test the validity of this conclusion. It was then observed that in the blue tetrazolium reaction x_1 showed marked reducing power, but the colour produced was only half as much as that given by an equivalent amount (based on the Kober colour produced) of 16-oxooestradiol-17β. The presence of 16α-hydroxyoestrone was therefore indicated. Further evidence was forthcoming when it was found that x_2 was mainly destroyed by alkali. Moreover, paper chromatography of the products obtained after alkaline treatment suggested that they might consist of unaffected 11β-hydroxyoestrone, an unknown substance with R_f intermediate between those of oestriol and 16-*epi*-oestriol and a major component, probably marrianolic acid, which remained at the origin. The reduction products of x_1 also gave a positive David reaction, thereby indicating the presence of/

of oestriol. It may be added that 11β-hydroxyoestriol-17β but not 6β-hydroxyoestradiol-17β also gives a green David colour. However, the amount of 11β-hydroxyoestradiol-17β present in the reduction products would not account sufficiently for the colour produced. It is therefore provisionally concluded that 11β-hydroxyoestrone and 16α-hydroxyoestrone were formed in the *in vitro* conversion of oestrone by ox adrenal homogenates.

Identification of x_2. The metabolite x_2 also gave a very weak Zimmermann reaction. Its reduction product did not yield more colour in the Kober reaction. On paper chromatography with the chloroform-formamide system the reduction product showed essentially one spot, which had nearly the same R_f as 16-*epi*oestriol, and hence was but slightly more 'polar' than its precursor (Fig. 1,V). This seemed to indicate that the two alcoholic hydroxyl groups were near enough for intramolecular hydrogen bonding, and, if this were so, it would appear that it might be 18-hydroxyoestradiol-17β derived from 18-aldo-oestrone by reduction. This is not inconsistent with/

with the chromatographic behaviour of x_2 which is less 'polar' than 18-hydroxyoestrone. Moreover, the failure to detect 18-hydroxyoestrone in the large-scale work (see below) might possibly be due to its further metabolic conversion. It was considered likely that 18-aldo-oestrone might give at the most a very weak Zimmermann reaction.

Since 1:3-diketones or, presumably, 1:3-dioxo compounds, undergo cleavage on treatment with alkali (Hauser, Swamer & Ringler, 1948), it might be expected that x_2, if it contained mainly 18-aldo-oestrone, would yield formic acid and an 18-noroestrone under similar conditions. The finding that x_2 on alkaline treatment yielded a very 'polar' product, presumably acidic in nature, instead of an 18-noroestrone, therefore suggested that it might contain an α-ketol grouping. This was subsequently shown to be so by its reduction of blue tetrazolium. The colour produced was nearly the same as expected for an equivalent amount of 16-oxooestradiol-17β. Moreover, the rate of reduction of blue tetrazolium was very rapid in contrast to that of x_1. Thus, the reduction product of x_2 was 16-<u>epi</u>oestriol, as also

also indicated by its giving a positive David reaction; x_2 is therefore 16-oxooestradiol-17β.

The 16-oxooestradiol-17β in x_2 must have been derived from 16α-hydroxyoestrone by re-arrangement of the latter in alkaline solution. The presence of 16α-hydroxyoestrone in x_1 was therefore indicated and was finally established (see above).

Isolation of a crystalline metabolite x_4 from the KC-6 fraction

Fractions 30-50 on evaporation yielded 14 mg. of oil, which contained 3 mg. of 'KC-6A.' This was arrived at by calculating from the observed intensity of the Kober colour produced and on the assumption that it contained KC-6A (i.e. 18-hydroxyoestrone). The remarkable increase in Kober chromogenicity (about 240%) after reduction with sodium borohydride was also consistent with the idea that it was a weak Kober chromogen. However, it did not give any formaldehyde after alkaline treatment and was recovered unchanged. Moreover, the reduction product unlike that of 18-hydroxyoestrone was as 'polar' as oestriol. The oil failed to crystallize.

After/

After a Girard separation the ketonic fraction weighed 5.6 mg. From this was obtained after purification 1.4 mg. of crystalline material which had m.p. 220-224°. The infrared spectral data showed that it is a mono-hydroxylated metabolite of oestrone, as was also evident from its chromatographic behaviour.

Probable structure of the crystalline metabolite, X_4.

The fact that both the hydroxyl groups could be acetylated (absence of free hydroxyl band in the infrared spectrum of the acetate) eliminates all the tertiary positions of the oestrone nucleus as possible sites of hydroxylation. Moreover, the presence of a band at 1377 $cm.^{-1}$ in the (KBr) spectrum of the free compound eliminates position 18. C-16 is also excluded on the infrared spectral evidence (band at 1408 $cm.^{-1}$) as well as results from chemical studies (see experimental section). The positions remaining for hydroxylation are therefore 6α, 6β, 7, 11α, 12 and 15.

6- and 11-Hydroxylation of neutral steroids are well documented in the literature; however, 11α-/

11α-hydroxylation has not been observed in ox adrenal homogenates and seems to be confined to microorganisms. It seems unlikely that 11α-hydroxylation of oestrone occurs in this instance. However, in view of the formation of a 11β-acetate from 11β-hydroxyoestradiol-17β (Magerlein & Hogg, 1957) under conditions in which esterification of the 11β-hydroxyl group of a neutral steroid is not feasible, the 11-position appears to be less hindered than in the neutral steroids and this might favour the enzymic introduction of an 11α-hydroxyl group into oestrone. Furthermore, an 11α-hydroxyl group in the oestrogen series would conceivably exert a more 'polar' effect than an 11β-hydroxyl, as was already observed in the neutral steroids (Savard, 1954). Hence, until evidence to the contrary is forthcoming, the 11α-configuration cannot be ruled out.

6β-Hydroxylation of oestradiol-$^{14}C_{16}$ by rat liver microsomes has been reported recently by Mueller & Rumney (1957). Though no authentic 6α- or 6β-hydroxyoestrone is available for a direct comparative study with the isolated product/

product, it seems unlikely that x_4 is 6β-hydroxyoestrone, because the absorption spectra of the methylated reduction product of x_4 and of 6β-hydroxyoestradiol-17β 3-methyl ether in concentrated H_2SO_4 differed significantly. 6α-Hydroxylation of androst-4-ene-3:17-dione has been demonstrated by Meyer, Hayano, Lindberg, Gut & Rodgers (1955) in ox adrenal gland homogenates, and, in agreement with the conclusions drawn by Savard (1954) the 6α-hydroxylated derivative was found in chromatographic fractions more'polar' than those containing the 6β-epimer. Hence x_4 might be 6α-hydroxyoestrone. However, the reduction product could not be resolved from 6β-hydroxyoestradiol-17β in the solvent systems used and thus might not be 6α-hydroxyoestradiol-17β. In addition, the presence of a band (shoulder with a NaCl prism; band with a CaF_2 prism) in the infrared (KBr) spectrum of x_4 at about 1438 $cm.^{-1}$, believed to be due to an unsubstituted methylene group adjacent to a double bond or to an aromatic ring (Jones & Cole, 1952) seems to militate against any oxygenation at C-6, though the exclusion or assignment of a structure by one spectral band is not entirely satisfactory.

Since/

Since 6β-hydroxylation in general occurs to a lesser extent than 11β-hydroxylation (Grant, 1956), and the 6β-hydroxylating activity of the adrenal gland is comparable to, if not greater than, that of 6α-hydroxylation, the finding that the yield of x_4 was so much higher than that of 11β-hydroxyoestrone, would seem to further exclude the 6α- configuration for the hydroxyl group.

Of positions 7, 12 and 15, the last is the least probable, since 15-hydroxylation has not been demonstrated in mammalian systems. It therefore appears that the metabolite might be hydroxylated at either 7 or 12. 7α-Hydroxylation has been achieved (e.g. Bergstrom & Gloor, 1954, 1955; Gloor, 1954) <u>in vitro</u> by using rat liver slices and homogenates, while the earlier demonstration of 7α-hydroxylation of deoxycholic acid in the rat by Bergstrom, Rottenberg & Sjovall (1953) is noteworthy. The presence of bile acids in ox bile points to the capacity of the liver to effect 7α- and 12α-hydroxylation. It remains to be seen whether or not the bovine adrenal possesses similar hydroxylating mechanisms/

mechanisms. In this connection it is interesting to note the isolation of a 7-oxo derivative of dehydro*epi*androsterone (DHA) from human urine (Fukushima, Kemp, Schneider, Stokem & Gallagher, 1954). Though artifact formation could not be definitely excluded, these workers believed that it was a normal metabolite derived from 7-hydroxy-DHA, since larger amounts were consistently observed in urines from patients with adrenal abnormalities.

A 7-hydroxyoestrone has been described by Pearlman & Wintersteiner (1940), but its reported melting point is about 40° higher than that of x_4. However, it is possible that they are epimeric at position 7. On the other hand, the infrared spectral evidence seems to favour a 12α-hydroxyl group in x_4. The C-O stretching absorption band at 1240 cm.$^{-1}$ of the alcoholic acetate group falls in the region as observed for the 12α-acetates of cholanic acid derivatives, viz., 1240-1242 cm.$^{-1}$ (Jones, Humphries, Herling & Dobriner, 1951). In the 12β-acetates this band occurs at 1232-1235 cm.$^{-1}$, while in the 7α-acetates at 1232-1237 cm.$^{-1}$.

Moreover/

Moreover, the weak Kober chromogenicity of x_4 might be accounted for by the location of the hydroxyl group at either 7 or 12. In this respect it may be mentioned that 11β-hydroxy-oestrone gives only about 10% colour in the Kober reaction, when compared with that produced by an equivalent amount of oestriol; this is presumably due to dehydration resulting in an olefinic bond between C-9 and C-11. The axial 12α-hydroxyl group might also be eliminated under the strong acid conditions of the Kober reaction, giving rise first to a 11,12-ethylenic bond, which would then rearrange to a 9,11-double bond. Dehydration of 7-hydroxyoestrone would result initially in the formation of an equilin or 1,3,5(10),6-oestratetraen-3-ol-17-one. It is therefore noteworthy that 17-dihydro-equilin gives about 33% Kober colour under the usual conditions. No observations have yet been made on the behaviour of 6-hydroxyoestrone or 1,3,5(10) 6-oestratetraen-3-ol-17-one in the Kober reaction. However, the observed Kober chromogenicity of x_4 (10%) is more in agreement with that of 11β-hydroxyoestrone than of equilin (33%).

However/

FIG. 4, V.

H_2SO_4 CHROMOGENS OF EQUILIN & 11β-HYDROXYOESTRONE.

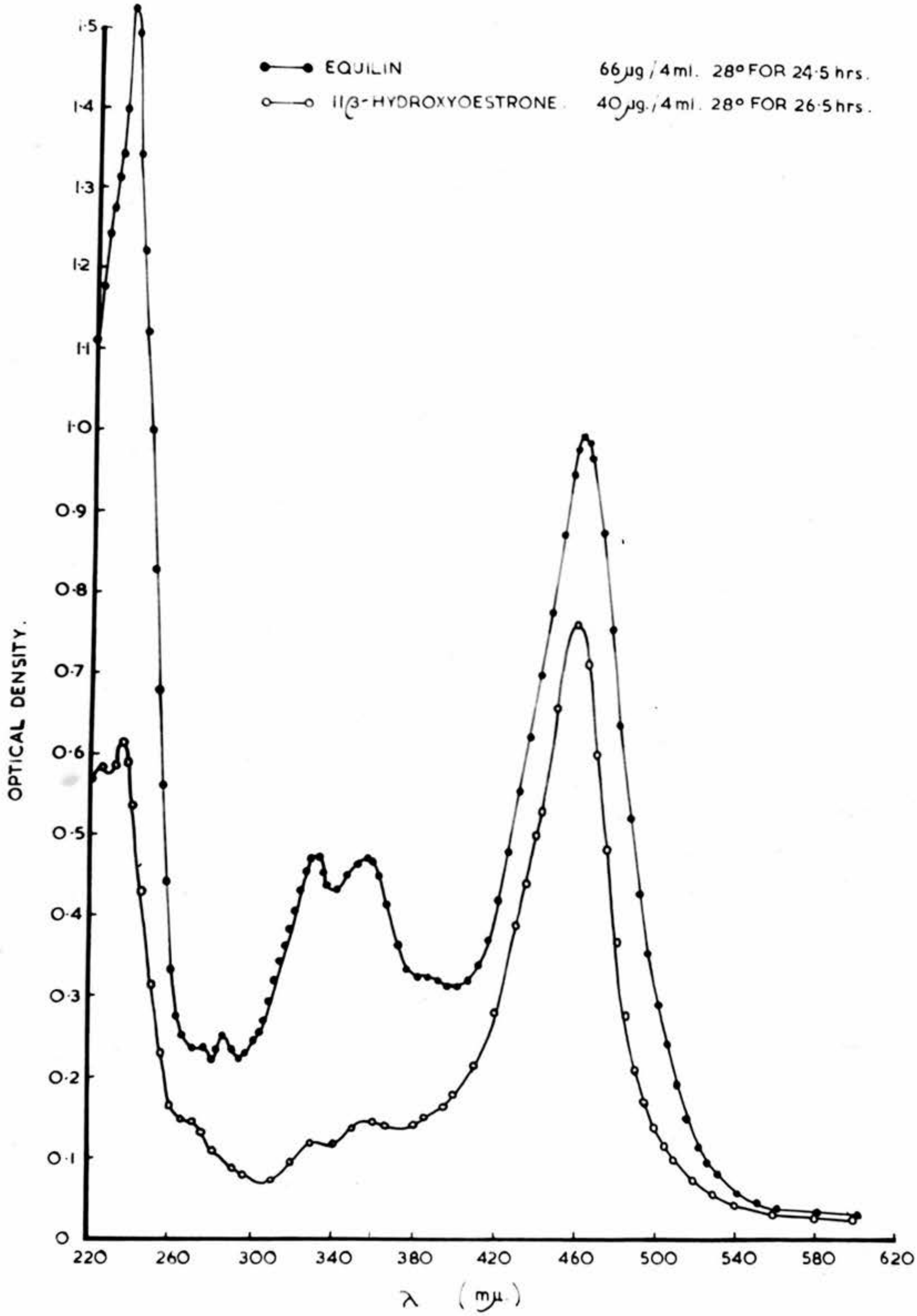


FIG. 3,V.

H_2SO_4 CHROMOGENS OF 11β-HYDROXYOESTRONE AND X_4.

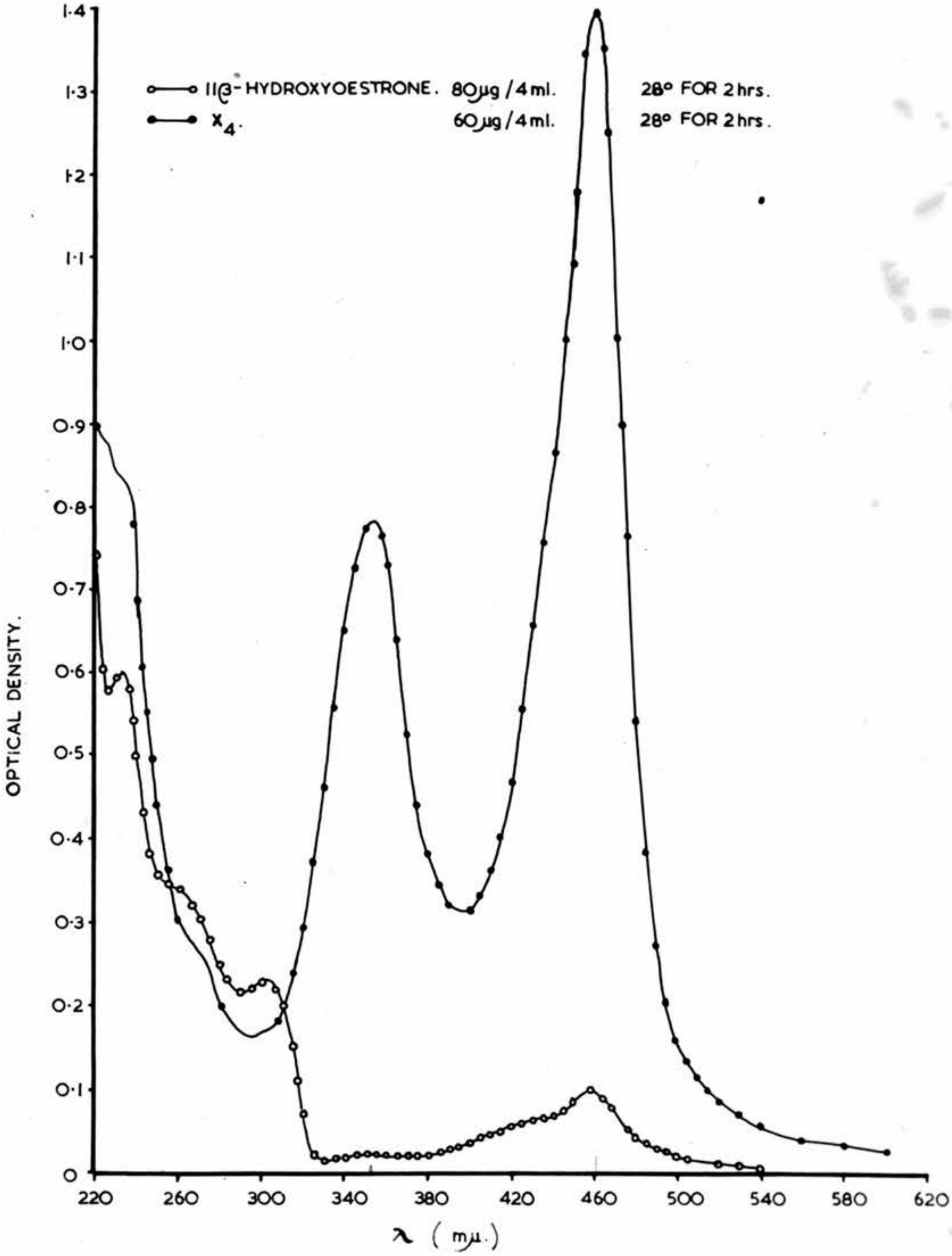
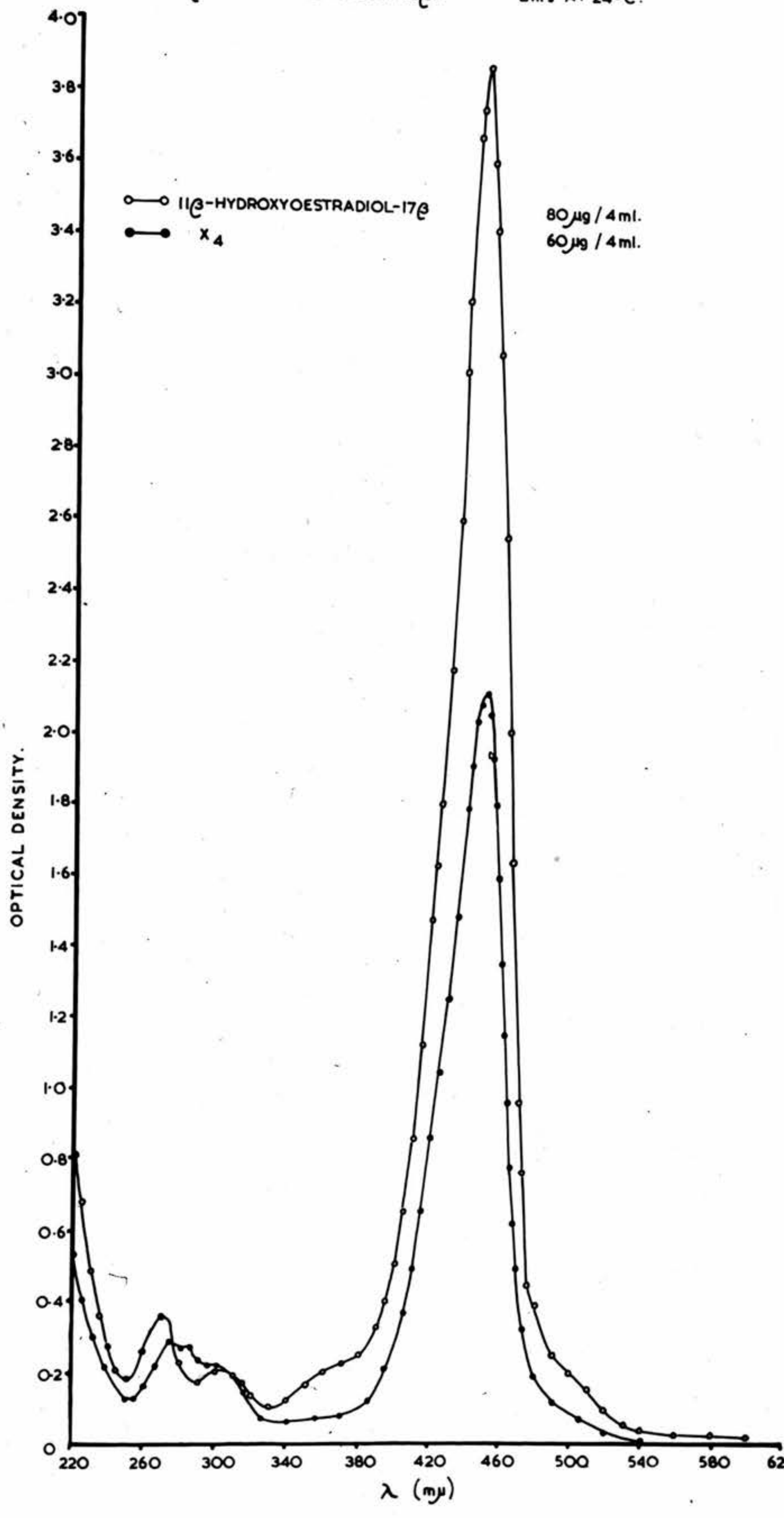


FIG. 2, V.

H_2SO_4 CHROMOGENS OF REDUCTION PRODUCT OF X_4 AND 11β-HYDROXYOESTRADIOL-17β. 2hrs AT 24°C.



However, the absorption spectrum of x_4 in concentrated H_2SO_4 seems to exclude position 7 as a possible site of hydroxylation, for it differs considerably from those of equilin and 11β-hydroxyoestrone (Figs. 3,V and 4,V). It will be noted (Fig. 4,V) that the spectra of the latter two compounds in H_2SO_4 are qualitatively the same. This might be expected since equilin is readily converted to 8,9- and 9,11-dehydro-14-isooestrone upon acid treatment (Banes, Carol & Haenni, 1950). Indicentally, this difference in the spectra of x_4 and 11β-hydroxyoestrone also appears to exclude the 11α-configuration, since by analogy with the finding of Rosenkrantz, Mancera & Sondheimer (1954), 11α-hydroxyoestrone might be expected to yield readily the 9,11-ethylene on dehydration.

On the other hand, the reduction product of x_4 had a spectrum not unlike that of 11β-hydroxyoestradiol-17β (Fig. 2,V). Though differing distinctly from that of 6β-hydroxyoestradiol-17β, it could not however be differentiated from that of 17-dihydroequilin with certainty. It would therefore appear that while militating against the 7-hydroxyoestrone structure, these results do not provide/

provide unequivocal support for the 12-hydroxyoestrone one for x_4.

However, a probable explanation for these apparently conflicting results obtained with the C-17 ketones and the 17β-hydroxyl derivatives may be as follows. It is possible that the 17β-hydroxyl might be more readily cleaved than a 12-hydroxyl. If such be the case, the ensuing pinacolic rearrangement would result in a migration of the C-18 methyl to C-17 (Cohen, Cook & Hewett, 1935), while subsequent dehydration of the 12-hydroxyl would follow the course postulated above with the eventual formation of a H_2SO_4 chromogen not unlike that derived from 11β-hydroxyoestradiol-17β. On the other hand, there is no competing pinacolic rearrangement in the dehydration of a 12-hydroxyoestrone. In consequence the migration of the C-18 methyl to C-12 might inhibit the formation of a 11:12-olefinic bond (presumably an essential step in the production of a chromogen similar to that of 11β-hydroxyoestrone), while at the same time lead to a different chromogenic structure.

Thus, on considering the available evidence it/

it seems reasonable to conclude provisionally that x_4 is 12-hydroxyoestrone, probably 12α. In this connection it may be mentioned that within the past eight months 12β-hydroxylation has been demonstrated for the first time in microorganisms (Schubert, Langbeim & Siebert, 1957; Guber & Tamm, 1958 a) as well as in mammalian systems (Brown, Wright & Okita, 1957). It remains to be seen whether x_4 is correctly formulated as such.

Experimental

The micro reactions carried out in this investigation have been described in previous sections. Data on the reference phenolic steroids are given in Appendix II.

(a) Preliminary small-scale experiments.

These experiments were scaled down to 1/100 of those described by Kahnt *et al*.

Homogenization - Ox adrenal glands were obtained in a fresh state and were preserved in a thermos flask packed with ice till ready for use. After removing the adhering fat, the adrenal glands were put through a chilled Latapie mincer and the pulp obtained was weighed. The following operations were carried out at 0°C. 6 G. of the pulp was homogenized in 6 ml. of sucrose solution for 2 minutes in a glass tube with a glass pestle; this was followed by another for 2 minutes in a glass tube with a nylon pestle. (The sucrose solution contained in one litre 68.4 g. sucrose, 3.62 g. NaCl, 3.56 g. $Na_2HPO_4.2H_2O$, 1.86 g. KCl, 1.16 g. fumaric acid and 3 g. nicotinamide and was brought up to pH /

pH 7.6).

Incubation - The homogenate was diluted with 6 ml. cane sugar solution, followed by 11 ml. of a fumarate solution (pH 7.5), which contained 3.62 g. NaCl, 3.56 g. $Na_2HPO_4\ 2H_2O$, 1.86 g. KCl and 4.68 g. fumaric acid in one litre. After adding 2.2 ml. 0.04 M-$MgSO_4$ solution, 3.2 mg. ATP, 0.32 mg. DPN and 0.1 mg. TPN, the pH was adjusted to 7.4. The final mixture was divided into two equal portions. One was used as a control (no oestrone added). To the other was added 6 mg. of oestrone in 0.6 ml. propylene glycol. The air in the incubation flasks was displaced with O_2, and the flasks were stoppered and shaken in a water bath at 37° C. for 3 hours.

Extraction and analysis of metabolites - The incubation mixture was diluted with 9 vol. of acetone and the precipitated proteins filtered off and extracted three times with 30 ml. of acetone. The combined acetone extract was evaporated to an aqueous residue under reduced pressure. Its volume was brought up to 15 ml. with/

with water and 35 ml. of methanol were added. After extraction of the aqueous methanolic solution with hexane (2 x $\frac{1}{2}$ vol.) to remove lipid material, the aqueous methanol phase was evaporated to a small volume under reduced pressure, and the aqueous residue was extracted twice with 50 ml. of ether. The ether extract was then extracted three times with 50 ml. of cold N-NaOH, each extract being immediately acidified with 5N-H_2SO_4. When cool, the acidified solution was then extracted three times with 60 ml. of ether, and the combined extract washed and evaporated to dryness.

The phenolic residue was separated into ketonic and non-ketonic fractions by means of the Girard reaction. The non-ketonic fraction was found to contain little or no Kober chromogens, and was not further investigated. A portion of the ketonic fraction containing before incubation 0.5 mg. of oestrone was chromatographed in a micro column. Fraction (0-12 ml.) which contained oestrone was assayed for recovery. The KC-5 fraction (16-36 ml.) was evaporated to dryness and the residue was treated with the Kober reagent. The/

The residue from the KC-6 fraction (38-60 ml.) was either examined by paper chromatography or treated with the Kober reagent. Since the yields were low, Kober reactions on 2 ml. fractions were not sensitive enough for detection.

Detection of KC-6 in incubation mixture.

When run on paper in the system methanol,75: water,35: benzene,70: chloroform, 40, the KC-6 fraction yielded a spot whose R_f was similar to that of 17-*epi*oestriol. The amount of metabolite was estimated visually by comparing the intensity of the Folin-Ciocalteu colour with that produced by 10 μg. of 17-epioestriol. A similar chromatographic fraction from the control showed no corresponding spot.

Preliminary identification of the metabolite in the KC-6 fraction as 18-hydroxyoestrone.

A portion of the incubation products estimated to contain about 10 μg. of the metabolite was chromatographed on Celite and the KC-6 fraction collected and evaporated to dryness. By following closely the procedure used in the micro scale alkaline/

alkaline degradation of 18-hydroxyoestrone (method b, Section IV, Experimental), the material after alkaline treatment was found to give about 0.3 μg. of formaldehyde (expected: 1 μg.) and a spot on paper chromatography similar to oestrone. A solvent control was carried out in the alkaline degradation test and gave negligible correction.

(b) Further small-scale experiments.

(i) ATP requirement - Glomerulosa tissue was obtained from the ox adrenal glands in the following manner. A gland was firmly held between two slabs and a thin slice was made on either side. A further slice would remove some zona fasiculata tissue. The glomerulosa layer of cells was scraped off from the capsule and the tissue thus obtained was minced. The pulp was then homogenized and the incubation mixture prepared as described in (a).

Using this source of hydroxylating enzymes, incubations were carried out to determine the ATP requirement. The combined fraction containing KC-5 and KC-6 from each of the two incubation mixtures was chromatographed on paper together with/

with 17-epioestriol. The intensities of the three overlapping spots were compared with one another. The yield (about 10%) was only slightly better in the presence than in the absence of ATP.

(ii) TPN requirement - These were performed with modifications by Grant & Brownie (1954). The glomerulosa tissue (6 g.) was homogenized in 12 ml. of 0.25 M-sucrose solution containing 0.12 M nicotinamide in the manner as described above. The homogenate was centrifuged at 700 g. for 5 minutes and the pellet thus obtained was re-suspended in 3 ml. sucrose solution and homogenized for 2 minutes in the tube with the nylon pestle. After centrifugation the supernatant was added to the main portion, the final volume of the preparation was about 15 ml. and was then mixed with 4 ml. TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer, pH 7.4, containing 6.5 mg. $MgSO_4.7H_2O$, and 4 ml. of potassium fumarate solution, pH 7.4). The final homogenate solution was then divided into two portions. To each were added 1 mg. of DPN and about 1.7 mg. of oestrone in/

in 0.5 ml. propylene glycol. The mixtures were then incubated for 3 hours at 37° under oxygen.

Using this procedure, the TPN requirement was investigated by adding 0.2 mg. to only one flask. The metabolites isolated in the usual manner were examined by paper chromatography. The total yield of the three metabolites was greater when TPN was present.

(iii) To investigate whether the zona glomerulosa or the zona fasiculata contains the higher hydroxylating activity.

Zona glomerulosa tissue was obtained as described previously. The fasiculata tissue was obtained by making further slicings of the adrenal gland, care being taken to avoid the medulla tissue. Equal weights (wet) of these two types of tissue were taken for the preparation of the homogenates which was carried out as described in b(ii). To the homogenates were added each 1 mg. DPN and 1.7 mg. of oestrone in 0.5 ml. propylene glycol. Incubation was carried out for 3 hours.

Again, three metabolites were obtained in each case. While the combined yield was higher with/

with glomerulosa than with fasiculata tissue, it was observed that the strongest spot (KC-6) in the glomerulosa was the weakest in the fasiculata and _vice versa_ in the case of the metabolite similar to 16α-hydroxyoestrone.

In a similar manner, it was shown that using the whole adrenal gland (cortex and medulla) the yield was much lower than that obtained with the cortex.

(c) Large-scale experiments.

A typical experiment was carried out as follows:-

The adrenal medullae were removed and the cortical tissue scraped off from the capsular material. The scrapings were minced and the pulp obtained weighed. About 32 g. of the pulp were suspended in 48 ml. of cold sucrose-nicotinamide solution and blended at half speed in a chilled Nelco blendor for 1 minute. The resultant brei was diluted with 18 ml. sucrose-nicotinamide solution and portions of this were homogenized in a glass tube with a nylon pestle for/

for 2 minutes. The homogenates were centrifuged at 700 g. for 10 minutes at 0° and the pellets obtained were combined and homogenized for another 2 minutes in fresh sucrose-nicotinamide solution (10 ml.). The final volume of the combined supernatants was about 64 ml. This was diluted with 32 ml. TRIS buffer, pH 7.4, containing Mg^{++} and 32 ml. potassium fumarate solution, pH 7.4. After adding 8 mg. DPN, the mixture was divided into 8 equal portions (ca 16 ml. each). To each portion in a 100 ml. Erlenmeyer flask was added 1.7-1.9 mg. of oestrone in propylene glycol (0.5 ml.). Incubation was carried out under oxygen for 3 hours at 37° C.

The mixtures were poured into 800 ml. acetone and the precipitated solids re-extracted three times with 180 ml. of acetone. The aqueous residue obtained after removal of acetone was diluted to 75 ml. with water. After adding 175 ml. of methanol, the lipid material was extracted twice with ½ vol. of hexane. The aqueous methanol phase was taken to a small volume (ca 70 ml.) and extracted with ether (1 x 200, 2 x 100 ml.). The ether extract was washed/

washed once with 20 ml. of water. After adding Bradosol to reduce emulsion formation, the ether phase was extracted with N-NaOH (3 x 180ml.). The phenols were recovered from the alkaline phase after acidification by extraction with ether (1 x 150, 3 x 125 ml.). The washed extract afforded a semi-crystalline residue on evaporation.

The phenolic residue was dissolved in methanol and an aliquot containing 150-200 μg. (based on added oestrone) was spotted on paper. In earlier work the Bush type of system was used; at times serious streaking took place; otherwise, satisfactory papergrams were obtained. Besides oestrone, three or four spots were detected. Three were the usual ones, the fourth being intermediate between 16α-hydroxyoestrone and oestrone was presumably oestradiol-17β.

When chromatographed in the chloroform-formamide system for 12 hours, both oestrone and oestradiol-17β were eluted from the paper, while the three spots obtained in the Bush type of system with the combined KC-5 and KC-6 fraction were/

were resolved into four (Fig. 1,Va).

The remaining material after removal of solvent was stored at 0°C.

Six such experiments were carried out, while another six were carried out with the incubation medium of Kahnt et al.

Isolation and Identification of Metabolites.

The combined phenolic residues weighed 318 mg. After crystallization from ethanol 128 mg. of oestrone was obtained. Concentration of the mother liquors and the washings afforded another 25.2 mg. of oestrone. The mother liquors were evaporated to dryness. The residue, 161 mg., was a coloured semi-crystalline oil. This was chromatographed on Celite as described in the isolation of KC-6A.

Fractions 1-6 which contained oestrone were not investigated.

Fractions 7-12 which contained 800 μg. of Kober chromogen gave on evaporation to dryness a semi-crystalline oil. The Kober chromogenic material entirely passed into the non-ketonic fraction after a Girard separation. Since oestradiol/

oestradiol-17β gives about 50% colour with the oestriol reagent, the amount actually present was about 1.6 mg. Volatile phenols were present in the fraction.

Fractions 13-20 on removal of solvent gave 12 mg. of oil. The ketonic fraction obtained from this was dissolved in 1 ml. of methanol and a 1/100th aliquot was spotted on paper impregnated with formamide. When developed with chloroform for 12 hours, no spot was obtained on spraying with the Folin-Ciocalteu reagent.

Fractions 21-29 yielded 9 mg. of oil. After a Girard separation the ketonic fraction weighed 4.4 mg. Paper chromatographic analysis in the chloroform-formamide system showed three spots, the most 'polar' of which being 'KC-6A' (see below) and corresponded to x_4 in Fig. 1,Va. These were separated on a preparative paper chromatogram, using the chloroform-formamide system. The zones containing the unknown metabolites, x_1 and x_2, x_1 being the less 'polar', were cut off and extracted four times with methanol. After removal of solvents, the liquid residue containing some formamide was partitioned between ether and water, and/

and the ether phase washed twice with fresh portions of water and evaporated to dryness. The residues when examined by paper chromatography were homogeneous and were indistinguishable from 16α-hydroxyoestrone and 16-oxooestradiol-17β respectively.

Identification of X_1.

The remaining material was dissolved in 5 ml. of methanol. A 0.1 ml. aliquot containing ca 17 μg. F-C chromogen yielded 3.2 μg. Kober chromogen calculated as oestriol. After reduction with $NaBH_4$ a similar aliquot was found to contain 3.7 μg. Kober chromogen. Paper chromatographic analysis of the reduction product in the chloroform-formamide system (22 hr.) revealed the presence of two spots indistinguishable from oestriol or 6β-hydroxyoestradiol-17β and 11β-hydroxyoestradiol-17β respectively (Fig. 1,Vb). The reduction product gave a positive David reaction.

A 0.5 ml. aliquot gave a very weak colour in the Zimmermann reaction.

Treatment of a 0.5 ml. aliquot with 0.5 ml.

2N-

2 N-NaOH for 24 hr. at room temperature afforded on paper chromatography unaffected 11β-hydroxyoestradiol-17β, an unknown product and material remaining at the origin (System: methanol,75: water,35: benzene,70: chloroform,40). The alkali-treated product showed a 70-80% loss in Kober chromogen.

In the blue tetrazolium reaction a 0.5 ml. aliquot containing ca 17 μg. Kober chromogen yielded a colour of optical density .030 (cf. .076 for ca 25 μg. of 16-oxooestradiol-17β). Visually there was a difference in the rates of colour development between the test and 16-oxooestradiol-17β.

Hence x_1 contained 16α-hydroxyoestrone and 11β-hydroxyoestrone.

Identification of x_2.

In a similar manner, a 0.1 ml. aliquot from a 5 ml. solution of x_2 showed little change in Kober chromogenicity after reduction with sodium borohydride (3.4 μg. $\longrightarrow$ 3.1 μg.).

The major reduction product had a mobility very similar to 16-epioestriol in the chloroform-formamide system (22 hr.)(Fig. 1,Vb). The reduction/

reduction product gave a weak David colour (about half of x_1).

Alkaline treatment of x_2 as described for x_1 afforded a product which was not eluted from the origin in the chloroform-formamide system. A Kober reaction on the remaining alkali-treated material revealed about 80% loss.

In the blue tetrazolium reaction a portion containing ca 17 μg. of Kober chromogen produced a colour of optical density .058 (cf. .076 for ca 25 μg. of 16-oxooestradiol-17β).

x_2 (1.5 ml.) produced only a weak colour in the Zimmermann reaction.

Hence x_2 contained predominantly 16-oxo-oestradiol-17β.

Preliminary experiments with KC-6 fraction.

Fractions 30-50 yielded 14 mg. of oil which failed to crystallize under the common organic solvents. 42 μg. of the crude material yielded only 1.7 μg. Kober chromogen calculated as oestriol. On reduction with sodium borohydride in methanolic solution, a product was obtained which/

which had the same R_f as oestriol in two solvent systems. The Kober chromogen content rose to 5.6 μg. after reduction (about 240% increase).

After treatment of 84 μg. of the crude incubation product (containing about 20 μg. of 'KC-6A') with N-NaOH (1 ml.) at room temperature for 4 hours, no formaldehyde was detected in the aqueous phase after acidification and extraction with chloroform (chromotropic acid test). The product recovered from the chloroform extract was unchanged material.

Isolation of crystalline metabolite x_4.

The oil was finally separated into ketonic and non-ketonic fractions. Nearly all the Kober chromogen was recovered in the ketonic fraction, weight, 5.6 mg. The oil partially solidified under ether, while chloroform dissolved this readily. By allowing the ether to evaporate spontaneously and triturating the partially solidified material with ether, crystals were obtained. After removal of the ether (pipe-stem filtration), 3.5 mg. of solid was obtained. This was leached with 0.15 ml. chloroform at -20°, yielding/

yielding 1.9 mg. of colourless product, m.p. 215-225° (hot stage). After crystallization from chloroform containing a trace of acetone, 1.4 mg. of fine colourless needles was obtained, m.p. 220-224° (yellow melt). Its absorption spectrum in concentrated H_2SO_4 was determined at 2 and 26 hours. In the Kober reaction crystalline x_4 gave about 10% colour.

Reduction with sodium borohydride was repeated on crystalline x_4 and essentially the same results recorded above were obtained. The reduction product was not affected in acid methanol and gave a David reaction. The absorption spectra of the reduction product and its 3-methyl ether in concentrated H_2SO_4 at 2 and 26 hours were determined. The reduction product like 11β-hydroxyoestradiol-17β produced a weak colour with a maximum near to that of oestradiol-17α in the Kagi-Miescher reaction.

Infrared spectral data of x_4.

The infrared measurements were kindly carried out by Dr A.E.Kellie of the Courtauld Institute of Biochemistry, London.

The/

The infrared spectrum (KBr) showed bands at 3380 cm.$^{-1}$ (alcoholic hydroxyl), 3270 cm.$^{-1}$ (phenolic hydroxyl), 1730 cm.$^{-1}$ (pentacyclic ketone), 1615, 1590 and 1503 cm^{-1} (aromatic ring), 1408 cm.$^{-1}$ (methylene group at C-16), and 1377 cm.$^{-1}$ (C-18 methyl).

The acetyl derivative, prepared by Dr Kellie, showed the following infrared spectral characteristics in CS_2 solution: (a) There was no free hydroxyl absorption band; (b) the C-17 ketone and the alcoholic acetate carbonyl absorbed at 1745 cm.$^{-1}$ while the phenolic acetate carbonyl absorbed at 1765 cm.$^{-1}$ (shoulder); (c) in the C-O region, bands at 1203 cm.$^{-1}$ and 1240 cm.$^{-1}$, characteristic of the phenolic acetate and the alcoholic acetate respectively, were present; intensities indicated that there was but one each of the acetate groups in the molecule (cf. comparison with spectra of oestrone acetate, oestradiol-17β diacetate and oestriol triacetate).

Discussion

In the large scale experiments the metabolites that have been provisionally identified on the evidence available are oestradiol-17β, 11β-hydroxyoestrone, 16α-hydroxyoestrone and 16-oxooestradiol-17β. A tentative structure has also been suggested for the only crystalline metabolite, x_4, isolated, namely, 12α(?)-hydroxyoestrone. Though 18-hydroxyoestrone has not been detected so far in the large scale experiments, it seems reasonable to assume its presence in the fraction containing x_4 in view of the results of the small scale experiments. For, its presence would not be revealed under the conditions of the alkaline degradation test (see Experimental), if it was present as a minor metabolite (say of the order of 11β-hydroxyoestrone). As will be seen later, other factors might also account for the failure to detect 18-hydroxyoestrone in the large scale work.

The/

The enzymic hydroxylations undergone by oestrone in the presence of ox adrenal cortical homogenates were therefore quite numerous. However, the yields, except for one metabolite, were all low. It is the writer's belief that much of this might be attributed to the age of the adrenals, the rather long time taken in working up the homogenates and perhaps the solubility of the substrate in the incubation mixture.

There is reason to believe that technical and other losses were not serious; indeed, these were surprisingly constant, for it was observed that oestrone reccveries in certain duplicate experiments (small scale) agreed to within 2% (average recovery: 85%). With the exception of 18-hydroxyoestrone and 16α-hydroxyoestrone, metabolites hydroxylated at the remaining positions of the oestrogen molecule are expected to be stable to alkali. Thus, losses arising from destruction by alkali would be very small. Though protein binding losses have been shown to be fairly serious in the case of oestriol, it is not so for oestrone. However, the possibility exists/

exists that the hydroxylated metabolites, being enmeshed, so to speak, within the enzyme protein, might not have been extracted quantitatively by acetone.

Finally, it must be emphasized that analysis of the incubation mixtures had been handicapped by the lack of a sensitive method. Except for 16α-hydroxyoestrone, most of the oestrone metabolites appeared to be weak Kober chromogens; hence the specific Kober reaction could not be used with advantage. It is therefore difficult to assess the individual hydroxylating activities of the ox adrenal gland.

With these reservations in mind, one might however conclude that under the conditions employed the hydroxylations fall in the following order of decreasing activity: 12α (?) $>$ 16α $\gg$ 11β $\geqslant$ 18.

Thus it seems that in contrast to the efficient 11β-hydroxylating activity of the ox adrenal gland shown towards the neutral steroids, 11β-hydroxylation occurs only to a very minor extent in the case of oestrone. It might be argued/

argued that the conditions used were suboptimal for 11β-hydroxylation since an adverse TPNH/TPN ratio,which had not been guarded against by the prior supplementation of TPN in these experiments, might arise when working with homogenates.

However, in the human species too, there seems to be a low level of 11β-hydroxylating activity with regard to oestrogens under normal circumstances. In a preliminary search for 11β-hydroxy derivatives of oestrone and oestradiol-17β in human pregnancy urine carried out in this Department, only small amounts (if these compounds were present at all) were detected chromatographically in an extract from several hundred litres of urine. It would therefore appear that a large and detectable output of 11β-hydroxy oestrogens in urine might be of diagnostic value in certain adrenal disorders.

Yet this low 11β-hydroxylating activity may well be a reflection of the fact that a steroid with its A ring distinctly different from those of the C-19 and C-21 neutral steroids, is presented to the adrenal gland hydroxylating enzymes. In consequence hydroxylation at other positions of the oestrogen nucleus, for example, C-12, might be/

be highly favoured. The dependence of enzymic hydroxylation upon the steric structure of the steroid-substrate has been recently pointed out by Guber & Tamm (1958a,b). Thus, while digitoxin underwent 12β-hydroxylation upon incubation with Fusarium lini (Bolley), androst-4-ene-3:17-dione, testosterone, progesterone and deoxycorticosterone all yielded the corresponding 15α-hydroxylated derivatives. No evidence of 12β-hydroxylation was obtained in the latter instances and in one case only was a di-hydroxylated derivative isolated, namely, 6β:15α-dihydroxyprogesterone.

Mention may be made of the metabolic x_8, which is identical to 16β-hydroxyoestrone in mobility in the chloroform-formamide system, and was detected on some of the analytical papergrams. However, there was no indication of its presence in either the final KC-5 fraction or the various non-ketonic fractions. It might be in the mother liquors of the KC-6 fraction. Nevertheless, one is tempted to consider the possibility that x_8 might be 16β-hydroxyoestrone, which finally appeared in the 16-oxooestradiol-17β fraction. Further work without the alkaline fractionation would/

would have to be done to settle this.

The failure to detect 18-hydroxyoestrone in the large-scale experiments was rather unexpected. However, destruction of this substance during the alkaline separation might account for this. Since one stage of the biosynthesis of aldosterone presumably involves oxidation of the 18-hydroxyl, the presence of an 18-dehydrogenase in the adrenal is to be expected. It remains to be seen whether a similar dehydrogenase exists, which would convert 18-hydroxyoestrone to 18-aldo-oestrone. If so, a further biological conversion of this nature might also be responsible for this failure to detect 18-hydroxyoestrone.

It is evident that further work is necessary not only for the complete identification of the various metabolites, but also to confirm or refute the results of the small-scale experiments in which 18-hydroxylation was demonstrated.

SECTION VI.

ISOLATION OF 2-METHOXYOESTRONE FROM THE URINE OF PREGNANT WOMEN

1. Introduction

Kraychy & Gallagher (1957a,b) have recently reported the isolation of 2-methoxyoestrone from the urine of human subjects treated with $[16-^{14}C]$ oestradiol-17β or $[16-^{14}C]$ oestrone. The identification was based on the complete identity between the compound isolated from urine and authentic 2-methoxyoestrone obtained by partial synthesis from oestrone.

Their observation was soon confirmed by Engel, Baggett & Carter (1957) who isolated 2-methoxyoestrone from the urine of a post-menopausal cancer patient treated with $[16-^{14}C]$ oestrone.

In pursuing our study of the mechanism of the Kober reaction it was hoped to investigate the Kober reaction of each new oestrogen derivative whenever available. Dr T.F. Gallagher very/

very kindly made available to us a specimen of 2-methoxyoestrone.

In the Kober reaction carried out by the procedure of Brown (1952) as modified by Bauld (1954), using the latter's 'oestriol reagent', 2-methoxyoestrone gave an immediate orange-pink colour in the first stage of the reaction which changed to red after 20 minutes' heating. The purple-pink colour obtained at the end of the second stage of the reaction showed a rather flat absorption maximum between 545 and 550 mμ. (cf. oestriol and oestrone, maximum at 512.5), and was readily distinguishable from the pink Kober colour produced by oestrone.

Subsequently, analysis of oestrone fractions from pregnancy urine revealed the presence of a compound producing a purple-pink colour in the Kober reaction in some of the early fractions. Since 2-methoxyoestrone is known to be less'polar' than oestrone, it was considered likely that this chromogen might be 2-methoxyoestrone itself. Further partition studies of the oestrone fraction soon established the presence of 2-methoxyoestrone in pregnancy urine and this compound/

FIG. 1. VI.

ABSORPTION SPECTRA OF KOBER COLOURS OF OESTRONE AND 2-METHOXYOESTRONE. (15 μg EACH IN 3·3 ml. SOLUTION.)

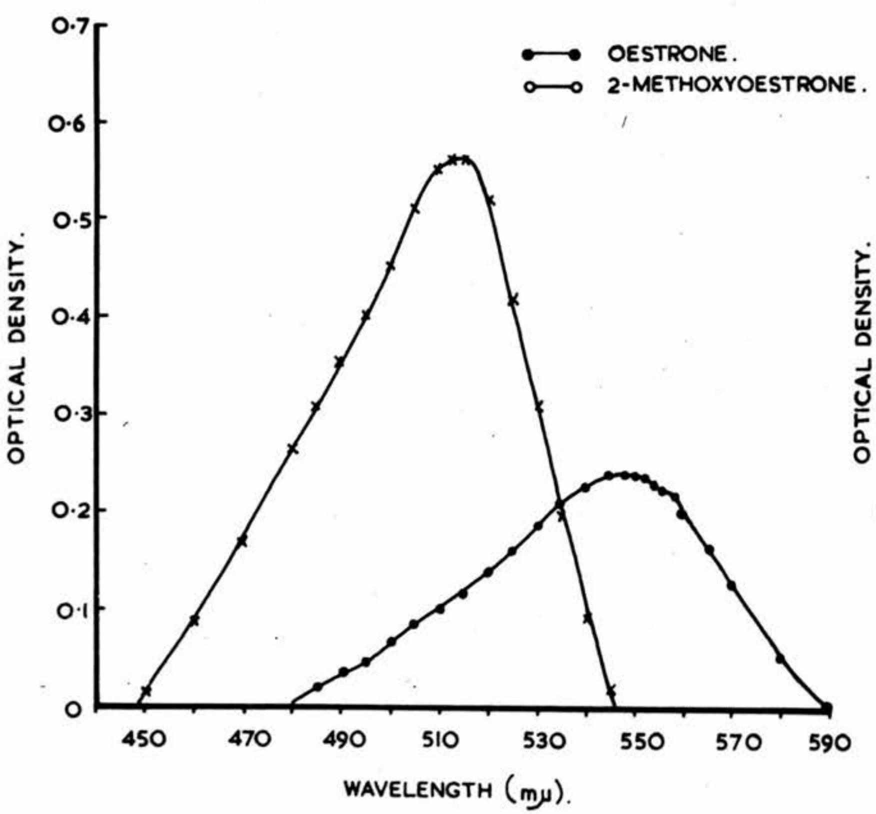
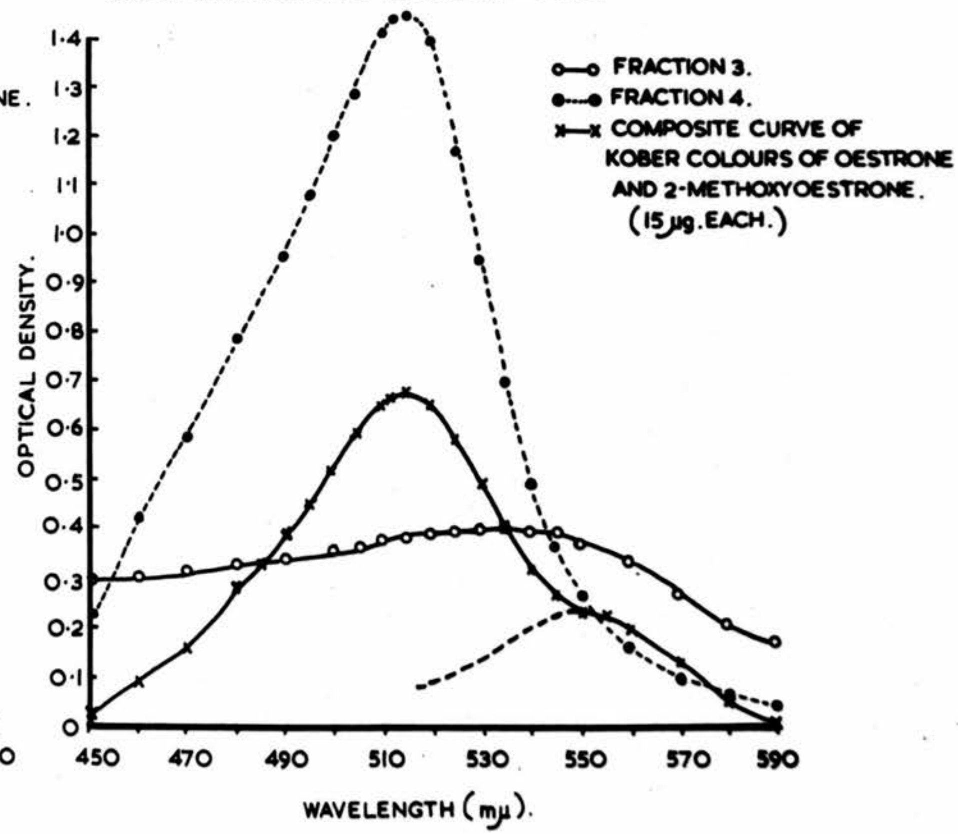


FIG. 2, VI.

ABSORPTION SPECTRA OF KOBER COLOURS OF CRUDE OESTRONE FRACTIONS FROM LATE PREGNANCY URINE.



compound was accordingly isolated.

2. Preliminary experiments

The following experiments were carried out on authentic 2-methoxyoestrone supplied by Dr T.F.Gallagher.

(a) Kober reaction

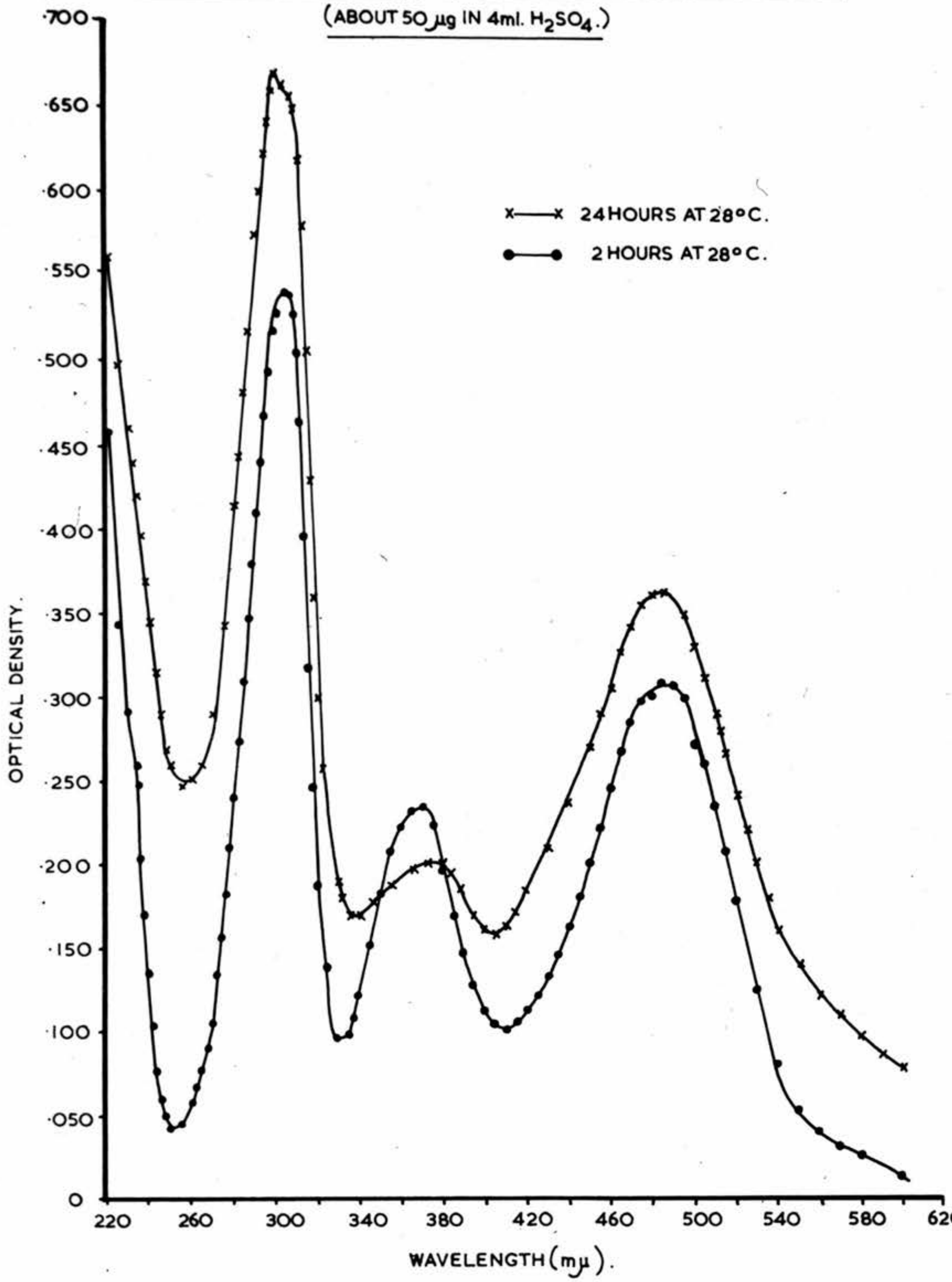
The absorption spectra of the Kober colour produced by 15 μg. each of 2-methoxyoestrone and oestrone are shown in Fig. 1.VI, and the composite curve obtained by adding the optical densities at the individual wavelengths, in Fig.2,VI.

The two wavelengths equidistant from the Kober absorption maximum of 2-methoxyoestrone at 545 mμ. , arbitrarily chosen for the Allen (1950) correction, are 512.5 and 577.5 mμ. The corrected optical density at 545 mμ. for 10μg. of 2-methoxyoestrone was found to be about 0.10 (compare the corresponding value of 0.260 for 10 μg. of oestrone). It is evident from the composite curve that the Allen correction is no longer applicable in the presence of oestrone or any urinary constituent which produces a strong colour at 510 mμ.

FIG. 3, VI.

ABSORPTION SPECTRA OF H_2SO_4 CHROMOGENS OF 2 METHOXY OESTRONE

(ABOUT 50 µg IN 4 ml. H_2SO_4.)



(b) H_2SO_4 chromogen

2-Methoxyoestrone, 57 μg., was dissolved in 4 ml. concentrated H_2SO_4. The solution was pale yellow with only weak fluorescence. After 20 minutes at 28° it became orange-red, which intensified slightly on prolonged standing. Fig. 3,VI shows the absorption spectra of the chromogen at 2 hr. and 24 hr. It appears that increases in the optical densities at 300 mμ. and 485 mμ. after the second hour were due to the gradual disappearance of the 375 mμ. maximum. It is noteworthy that the curve at 2 hr. is similar qualitatively to that of 16α-hydroxyoestrone, despite their different Kober chromogenic properties.

(c) 2-methoxyoestradiol-17β

Micro quantities of 2-methoxyoestradiol-17β were prepared by reduction of 2-methoxyoestrone with sodium borohydride in methanol as described in Section IV. The unpurified product gave the same absorption maximum in the Kober reaction. The corrected optical density at 545 mμ. was about equal to that of an equal amount of the parent ketone.

(d)/

(d) <u>2-methoxyoestrone-3-methyl ether</u>

The 3-methyl ether prepared from 2-methoxyoestrone according to the general method described in Section IV had a sharp maximum at 550 mμ and the intensity was about the same as that produced by an equivalent amount of the free phenol. It thus appears that the bathochromic shift of the Kober maximum at 512.5 mμ, typical of the oestrogens, may be entirely attributed to the introduction of a methoxyl group at position 2.

(e) <u>Separation of 2-methoxyoestrone from oestrone on micro partition columns</u>

While adequate separation of 2-methoxyoestrone from oestrone was obtained in paper chromatography using the system 70% methanol in water/benzene-hexane (1:1, by vol.), chromatography on a micro Celite column using the same solvent system resulted in partial resolution. The Kober chromogens were recovered in the first 14 ml.

The system 70% methanol in water/benzene-hexane (3:7, by vol.) was subsequently found to give good separation of the two compounds: 2-methoxyoestrone (2-12 ml.); oestrone (18-38 ml.).

3. /

3. Detection of 2-methoxyoestrone in the urine of pregnant women

A phenolic-ketonic fraction from 264 l. of enzymically hydrolysed late pregnancy urine was chromatographed on a 120 g. Celite column in the system 70% methanol in water/benzene-hexane (4:1, by vol.). On analysis of the various fractions by means of the Kober reaction, it was observed that there was a purplish-pink component in the Kober colours obtained with fractions 3 and 4. The absorption spectra of these colours are recorded in Fig. 2,VI.

The presence of a 2-methoxyoestrone-like Kober chromogen in the oestrone fraction (fractions 1-8 from the large chromatogram) was clearly demonstrated when a small portion of the combined oestrone fraction was chromatographed on a micro column as described in 2(e). It was estimated that there was 5.65 mg. of this Kober chromogen in the oestrone fraction from 264 l. of urine.

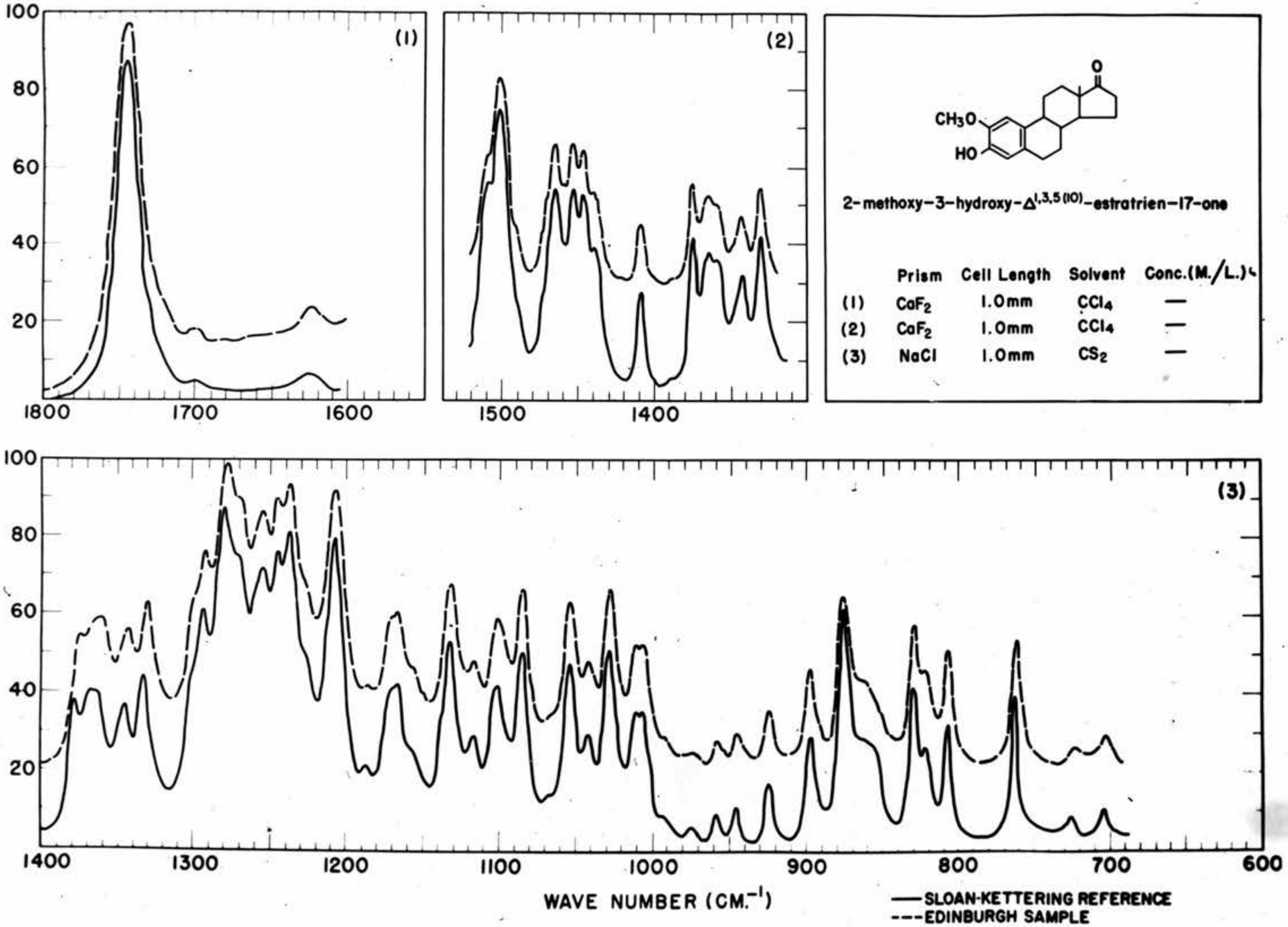
4. /

4. <u>Isolation of 2-methoxyoestrone from pregnancy urine</u>

An oestrone fraction from 700 l. of pregnancy urine was chromatographed on a Celite column using the system 70% methanol in water/ benzene-hexane (3:7, by vol.). Successive 40 ml. fractions were collected. Colorimetric analyses on aliquots from the various fractions showed that the 2-methoxyoestrone-like material was concentrated in fractions 6-8, while oestrone appeared in fractions 14-28. The amount present was about 16 mg.

Fractions 6-8 were combined and evaporated to dryness under reduced pressure. The brown oil, 94 mg., was dissolved in 0.2 ml. warm acetone and the solution set aside at -20°. Eighteen mg. of coloured crystals were obtained. Leaching this with fresh acetone afforded about 10 mg. of a white crystalline substance. This gave a negative Kober reaction and was not further investigated. The material soluble in cold acetone was chromatographed on 5 g. alumina (acid-washed; activated by heating at 140°). Discontinuous gradient elution was carried out with benzene-hexane, benzene, and acetone-benzene. Successive/

FIG. 4, VI. INFRA-RED SPECTRA OF ISOLATED MATERIAL AND AUTHENTIC 2-METHOXYOESTRONE.



Successive 10 ml. cuts were made and the fractions richest in the 2-methoxyoestrone-like Kober chromogen were combined and recrystallized twice from methanol at -20°. A white crystalline product, 2.1 mg., was obtained. Its melting point (uncorr.) was 183-184°. A further 2.1 mg. of material, m.p. 182-183°, was obtained from the mother liquors. Comparison of the isolated material with authentic material was kindly carried out by Dr T.F. Gallagher of the Sloan-Kettering Institute, New York. Fig. 4,VI shows conclusively the identity of the isolated material with authentic 2-methoxyoestrone.

5. Discussion

The presence of 2-methoxyoestrone in pregnancy urine is not surprising in the light of the metabolism studies of Kraychy & Gallagher (1957a,b) and Engel, Baggett & Carter (1957), for there is no reason to suppose that certain pathways of oestrogen metabolism are restricted to cancer patients. There may be, however, quantitative differences in oestrogen metabolism between normal and cancer patients.

From/

From the present work and the studies mentioned above it would appear that 2-methoxyoestrone is of some importance quantitatively. Using the Kober colorimetric assay, the amount of 2-methoxyoestrone in a litre of pooled pregnancy urine was about 20 μg. There is some indication that the Kober reaction for less than 5 μg. of 2-methoxyoestrone does not yield the colour as expected if Beer's Law is obeyed. Add to this, the presence of urinary contaminants that might interfere with the colour reaction, it will then be evident that the estimation by the Kober reaction is by no means quantitative. Moreover, some losses were inevitable during the extraction and the fractionation of the urines in large-scale work. Thus, it might be that the amount excreted is equal to, if not greater than, that found for oestradiol-17β.

It seems probable that the (x) Kober chromogen absorbing maximally between 538 and 548 mμ., detected by Smith & Blackham (1957) in extracts of pregnancy urine after Zn-HCl hydrolysis, but not in the absence of Zn, was derived from 2-methoxyoestrone. These workers used/

used Brown's method, which involves methylation of the oestrone-oestradiol-17β fraction and subsequent chromatographic separation on alumina. As reported above, methylation of the 3-phenolic group of 2-methoxyoestrone does not result in any great shift of the Kober maximum from 545 mμ though the introduction of a methoxyl group at position 2 shifts the 512.5 mμ. maximum, which is characteristic of the oestrogens, to 545 mμ. From the chromatographic behaviour of the (x) chromogen as reported by these workers, it appears probable that the (x) Kober chromogen may have been the 3-methyl ether of 2-methoxy-oestradiol-17β, which was produced from 2-methoxy-oestrone by chemical reduction during hydrolysis.

The biogenesis of 2-methoxyoestrone has been discussed by Kraychy & Gallagher (1957). It is concluded in the light of other instances of <u>in vivo</u> methylation of oxygen by man and animal (references cited by these workers) that the biological conversion of oestrone (or oestradiol-17β) to 2-methoxyoestrone is accomplished in two separate stages, i.e. oxidation and subsequent methylation.

The/

The immediate metabolic precursor of 2-methoxyoestrone is therefore 2-hydroxyoestrone. Niederl & Vogel (1949) have reported the partial synthesis of this compound from oestrone. However, in view of the recent work of Werbin & Holoway (1956) on the nitration of oestrone, it seems certain that Niederl & Vogel assigned the nitro group to position 2 instead of 4 and had actually prepared 4-hydroxyoestrone. Mueller & Mills (work mentioned by Mueller, 1955) have also prepared 2- and 4-hydroxyoestradiol-17β, but the work has not yet been described in detail.

2-Hydroxyoestrone is probably the first product to be formed in the ring A degradation of oestrone. It may be recalled that early workers on the inactivation of oestrogens by mammalian livers (for instance, see Pearlman, 1948; Lieberman & Teich, 1953) had long sought without success to obtain an enzyme akin to plant tyrosinase in activity. However, it is hoped that with present-day analytical techniques and the availability of synthetic oestrogen derivatives, re-examination of this problem might produce evidence that oxidative rupture of/

of the A ring of the oestrogens is one of the numerous inactivation processes occurring in the liver.

It would therefore appear that methoxylation of an oestrogen (or methylation of an oestrogen catechol) is but a side reaction in the main stream of events leading to the final breakdown of the oestrogen moelcule. Yet the interesting finding of Mueller (1955) seems to suggest otherwise. It was found that while the addition of oestradiol, oestrone or oestriol to surviving uterine segments failed to influence the uptake of labelled formate into protein, the 2-hydroxy and the 4-hydroxy derivative of oestradiol gave a significant 'oestrogen-like' stimulation to this process. It was also shown that the radioactive tag resided mainly in the serine and methionine residues of the protein. As was pointed out by Mueller (1957), it remains to be seen whether or not the other metabolic pathways for 'one carbon' fragments are stimulated in a manner typical of oestradiol <u>in vivo</u>. Speculative though they must be, two questions which have a bearing on the probable biogenesis/

biogenesis and the physiological role of 2-methoxyoestrone, might be asked.

The first is whether or not a 2-methoxy derivative is formed concomitantly when a 2-hydroxy oestrogen is carrying out the biological function mentioned above. And, secondly, whether or not the 2-methoxy derivative itself takes part in one-carbon transfer. In this connection it is interesting to note the weak oestrogenic activity shown by the ring A modified oestrogen derivatives. Thus, in the uterine growth test 2(?)-hydroxyoestrone and its 3-methyl ether possess negligible activity when compared with oestrone (Niederl & Vogel, 1949). The following table contains additional data pertaining to the oestrogenic activities of the ring A hydroxylated and methoxylated derivatives.

Table 1, VI /

Table 1.VI. Oestrogen activities of ring A hydroxylated and methoxylated oestrogen derivatives.

Compound	Oestrogenic activity (compared with oestradiol-17β)	References
Oestradiol-17β	1	-
2-Methoxyoestrone	1/20,000	Kraychy & Gallagher, (1957,b)
2-Hydroxyoestra-diol-17β	1/100	Mueller (1955)
4-Hydroxyoestra-diol-17β	1/10	Mueller (1955)

Though the comparison might not be strictly valid, the following order of decreasing activity seems to hold, viz., oestrogen $>$ 2- or 4-hydroxy derivative $>$ 2-methoxy derivative. Thus, with a progressive alteration of the oestrogen structure, there arises a corresponding fall in oestrogen activity. On the other hand, it may well be that this is parallelled by a rising trend in activity of a different nature, for/

for example, in the metabolism of one carbon units.

However, there is no reason to believe that a methyl group attached to oxygen is labile under biological conditions like, for instance, that of methionine. The fact that numerous O-methylated compounds have been isolated from urine in recent years (for example, DeEds, Booth & Jones (1957) and references cited by Kraychy & Gallagher) lends support to this. However, it may be that by virtue of its basic oestrogenic structure, 2-methoxyoestrone loses its methyl group as readily as it acquires it. It might be possible to test the stability of the methyl group by, for example, simulating the _in vitro_ experiment of Mueller, using instead 2-methoxyoestrone with a ^{14}C label in the methyl group and unlabelled formate.

Finally, the O-methylation of 2-hydroxyoestrone can, it is believed, now be studied under _in vitro_ conditions. For, recently, Axelrod (1957) has demonstrated the presence of an enzyme in the soluble supernatant fraction of rat liver, which can effect the O-methylation of several/

several catechols, e.g. epinephrine, in the presence of ATP and methionine, or, more efficiently, of S-adenosyl methionine. Perhaps it may then be feasible to elucidate another interesting and almost universal feature of O-methylation, i.e. the apparently specific methylation of the newly-introduced hydroxyl group.

SECTION VII. SEARCH FOR NEW OESTROGEN KETONIC METABOLITES IN PREGNANCY URINE.

1. Introduction

With the isolation of two more oestrogen metabolites, namely, 18-hydroxyoestrone and 2-methoxyoestrone, from human pregnancy urine, the diversity of the metabolic transformations undergone by the primary hormone, oestradiol-17β (or oestrone, by virtue of their interconvertibility), hitherto suspected, has thus been clearly demonstrated. Indeed, the isolation of 18-hydroxyoestrone, the first steroid outside the class of adrenocortical compounds shown to possess an oxygenated C-18 group, has revealed a close parallelism between the metabolism of the oestrogens and that of the neutral steroids. For, no longer needs metabolic transformations be confined to positions 16 and 17 of the oestrogen nucleus, but oxygenation at other positions, say 6 and 11, which has already been demonstrated in the other steroid hormones, might well/

well be expected.

In another way, too, that certain metabolic pathways may exist in both the aromatic and non-aromatic steroids is brought into focus. Thus, in isolating 16α-hydroxydehydro<u>epi</u>androsterone from male urine, Fotherby, Colas, Atherden & Marrian (1957) have, so to speak, bridged the metabolic gap between dehydro<u>epi</u>androsterone (DHA) and androst-5-ene-3β:16α:17β-triol. In the oestrogen field the isolation of 16α-hydroxy-oestrone - the probable intermediate between oestrone and oestriol - was soon followed up by the demonstration of its metabolic reduction to oestriol under *in vivo* conditions in man (Brown & Marrian, 1957). It might therefore be expected that the DHA analogue of 16-*epi*oestriol would eventually be found in male urine. This has indeed been shown to be the case recently (Fotherby, 1957).

6β-Hydroxylation of progesterone and oxidation of the 6β-hydroxyl to the 6-oxo group have been demonstrated under *in vitro* conditions (Berliner & Salhanick, 1956; Hagopian, Pincus, Carlo/

Carlo & Romanoff, 1956). The isolation of 6β-hydroxycortisol from human urine has been reported (Burstein, Dorfman & Nadel, 1954). It therefore appeared that 6-oxygenated oestrogen metabolites might also be formed and eventually excreted in the urine. The recent demonstration of the conversion of oestradiol-17β -16-^{14}C to 6β-hydroxyoestradiol-17β- 16-^{14}C and 6-oxo-oestradiol-17β- 16-^{14}C (Mueller & Rumney, 1957) further substantiated this belief.

6-Oxooestradiol-17β and its diacetate give a negative Kober reaction, but during the first stage of the reaction each develops an immediate orange colour which soon changes to red and subsequently fades away. On the other hand, 6β-hydroxyoestradiol-17β, though behaving somewhat similarly to 6-oxooestradiol-17β in the initial colour development, gives a stable colour at the end of the Kober reaction.

It was observed that chromatographic fractions between oestrone and KC-5 (i.e. fractions 9-14) gave colour changes similar to those of 6-oxooestradiol-17β in the Kober reaction. Though other urinary constituents might conceivably behave/

behave in a similar fashion, it was considered desirable to make a deliberate search for 6-oxo-oestrone (or, less likely, 6-oxooestradiol-17β) in these fractions. Other dioxo oestrogen derivatives like 16-oxooestrone and 11-oxooestrone, if they were present at all, would also occur in this fraction.

The detection of two Kober chromogens with absorption maximum at about 545 mμ is described in this section. For convenience, the term '545 mμ or 512.5 mμ Kober chromogen' is used. These 545 mμ Kober chromogens are believed to be the 2-methoxy derivatives of 16-oxooestradiol-17β and 16α-hydroxyoestrone, the former being partly formed artifactually by rearrangement of the latter. In addition, two 512.5 mμ Kober chromogens were detected in the concentrate from fractions 9-14 after reduction with sodium borohydride, one of which has been identified as oestriol. Their probable origin is discussed.

2. Preparation of concentrates from fractions 9-14 which presumably contained 6-oxooestrone

Fractions 9-14, obtained from ~~the~~ partition chromatography of a ketonic phenolic extract from 204 l. of enzymically hydrolysed pregnancy urine in/

in the system 70% methanol in water/benzene-hexane (4:1, by vol.) were combined to yield 130 mg. of oil. This was then transferred to an alumina column (acid-washed; activated by heating at 140°) prepared in benzene. Elution with benzene yielded a small amount of oil. Discontinuous gradient elution was then carried out with increasing quantities of acetone in benzene. The compound, which gave an immediate red colour in the first stage of the Kober reaction, was eluted from the column with 6-11% acetone in benzene. Fractions which gave the colour characteristic in the Kober reaction were combined to yield 17 mg. of oil. This was combined with another batch of material obtained in a similar manner from about 500 ml. of urine. The combined concentrate weighed 67 mg.

3. Preliminary studies on the concentrate

In the Kober reaction a 50 μg. portion produced a colour equivalent to 0.5 μg. of oestriol. There was no increase in Kober colour after reduction with $NaBH_4$. On chromatographing the reduction products and the/

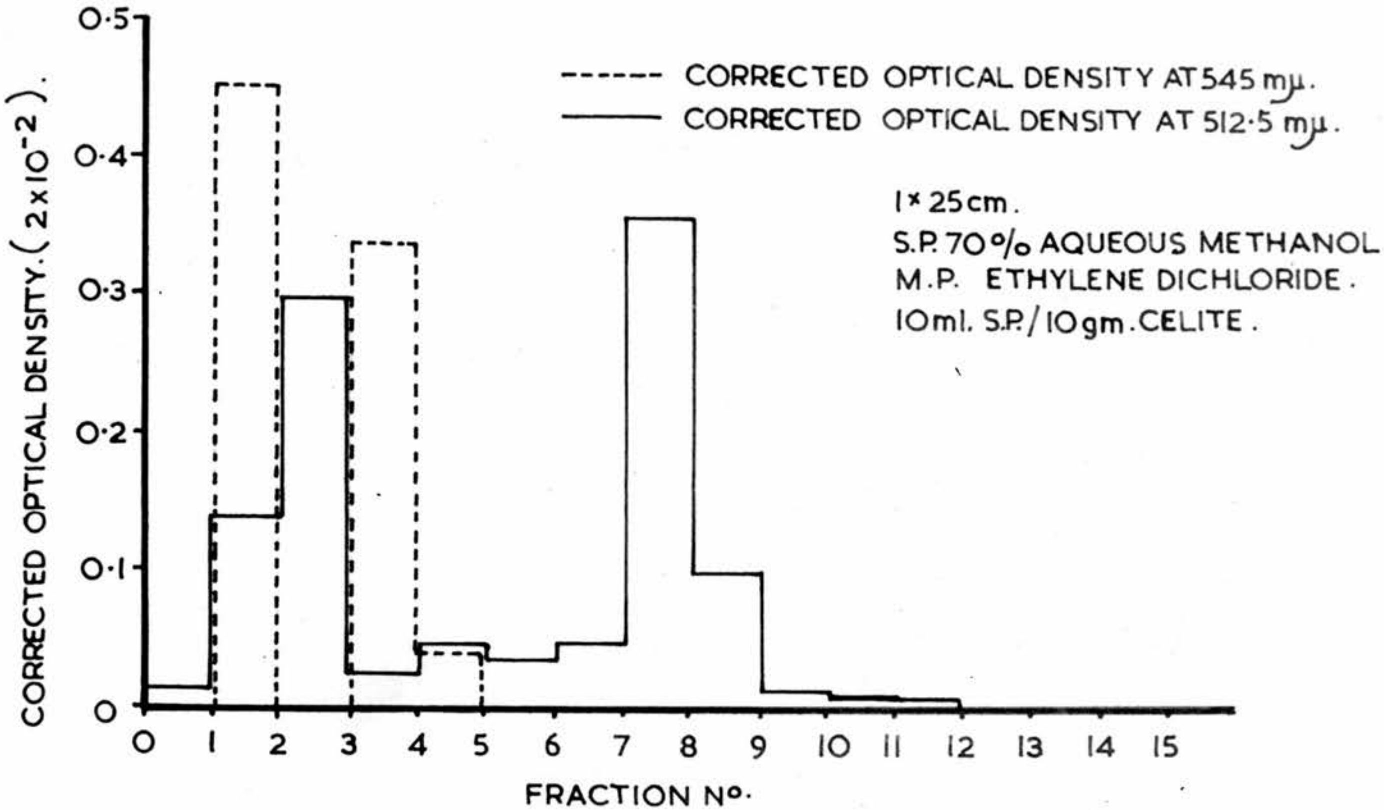
the starting material on paper in the system methanol,75; water, 35; benzene, 70; chloroform,40, and spraying the dried paper after development with the Folin-Ciocalteu reagent, three more weak spots were detected, besides a large spot which had the same R_f as the starting material. Two of the three more 'polar' spots had R_f's similar to oestriol and 16-epioestriol respectively, the remaining one being just ahead of the 'oestriol' spot. Visual examination of the intensities of the 'oestriol' and '16-*epi*oestriol' spots showed the presence of about 2% material in the total fraction.

In view of the small amounts of these hypothetical oestrogen metabolites present in the final concentrate, it was felt that isolation would not be feasible at this stage, and that a preliminary study of the reduction products might provide some light on the structure of these compounds. It may be mentioned that after sodium borohydride reduction no immediate red colour was observed in the Kober reaction.

4. /

FIG. I. VII.

CHROMATOGRAM OF THE REDUCTION PRODUCTS OF THE FRACTION BETWEEN OESTRONE AND KC-5 FROM PREGNANCY URINE.



4. <u>Reduction of the crude concentrate with sodium borohydride</u>

To a solution of the crude concentrate in 2 ml. methanol were added 150 mg. of sodium borohydride. After 17 hr. at room temperature, the reaction mixture was diluted with acid and ether, and the ether phase was separated, washed thrice with saturated NaCl solution and evaporated to dryness.

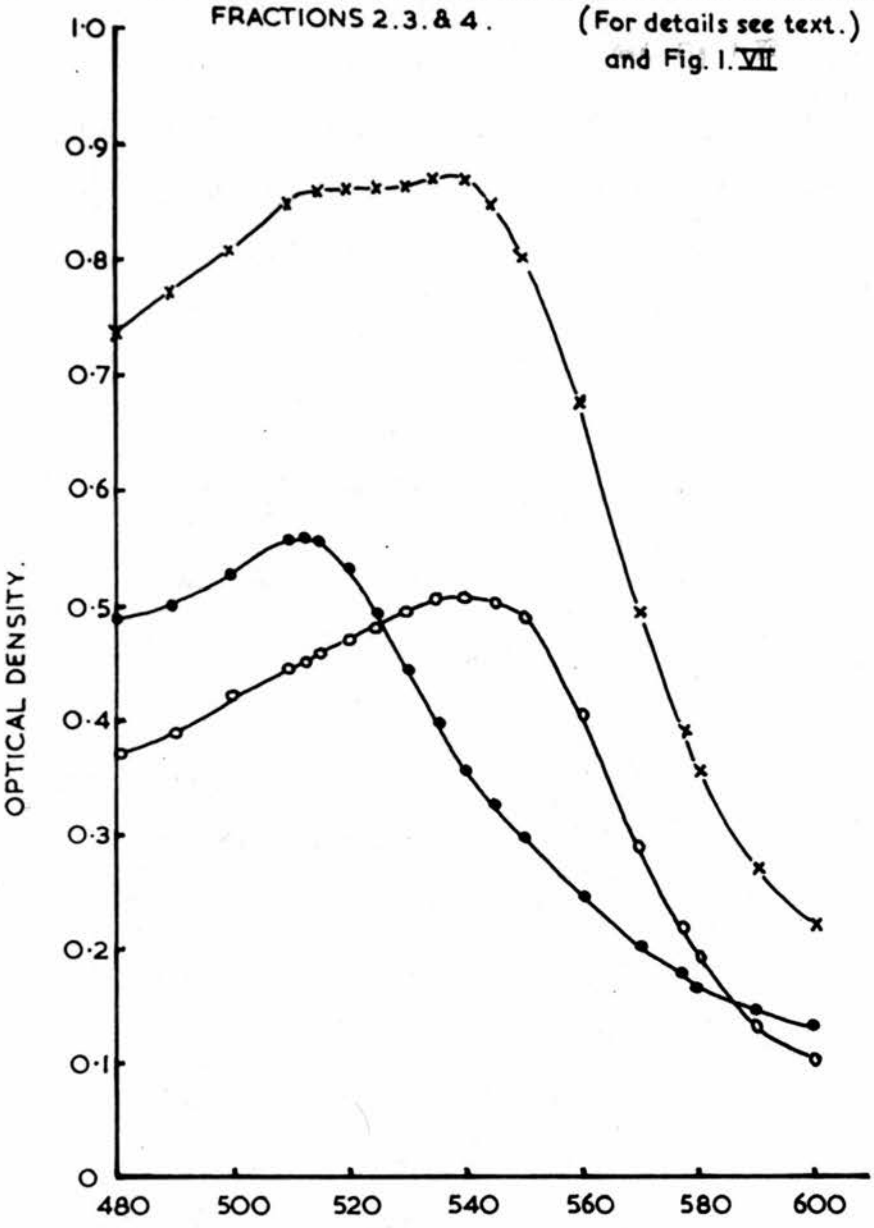
5. <u>Column chromatography of the reduction products</u>

The crude product obtained above was chromatographed on 10 g. of Celite, using the system 70% methanol in water/ethylene dichloride. Successive 5 ml. fractions were collected. One-fiftieth aliquots were removed for colorimetric assay. Fig. 1,VII shows the elution pattern of the Kober chromogens, while Fig. 2,VII the absorption spectra of the fractions 2, 3 and 4.

It will be noted that there were at least two Kober chromogens with absorption maximum at 512.5 mμ which resembled 16-<u>epi</u>oestriol and oestriol in mobility respectively. The 545 mμ Kober chromogens which were found in nearly equal amounts in the 16-<u>epi</u>oestriol fraction were /

FIG. 2, VII.

ABSORPTION SPECTRA OF KOBER COLOURS OF CHROMATOGRAPHIC FRACTIONS 2. 3. & 4. (For details see text.) and Fig. I. VII



were provisionally identified as 2-methoxy-16-*epi*oestriol and 2-methoxyoestriol on the basis of their chromatographic behaviour. Their precursors by analogy with the KC-5 problem would be 2-methoxy-16-oxooestradiol-17β and 2-methoxy-16α-hydroxyoestrone respectively. The 2-methoxy-16α-hydroxyoestrone structure for the precursor of the more 'polar' 545 mμ. Kober chromogen received further support in the following experiments.

6. Attempted isolation of 2-methoxyoestriol and the more polar 512.5 mμ Kober chromogen

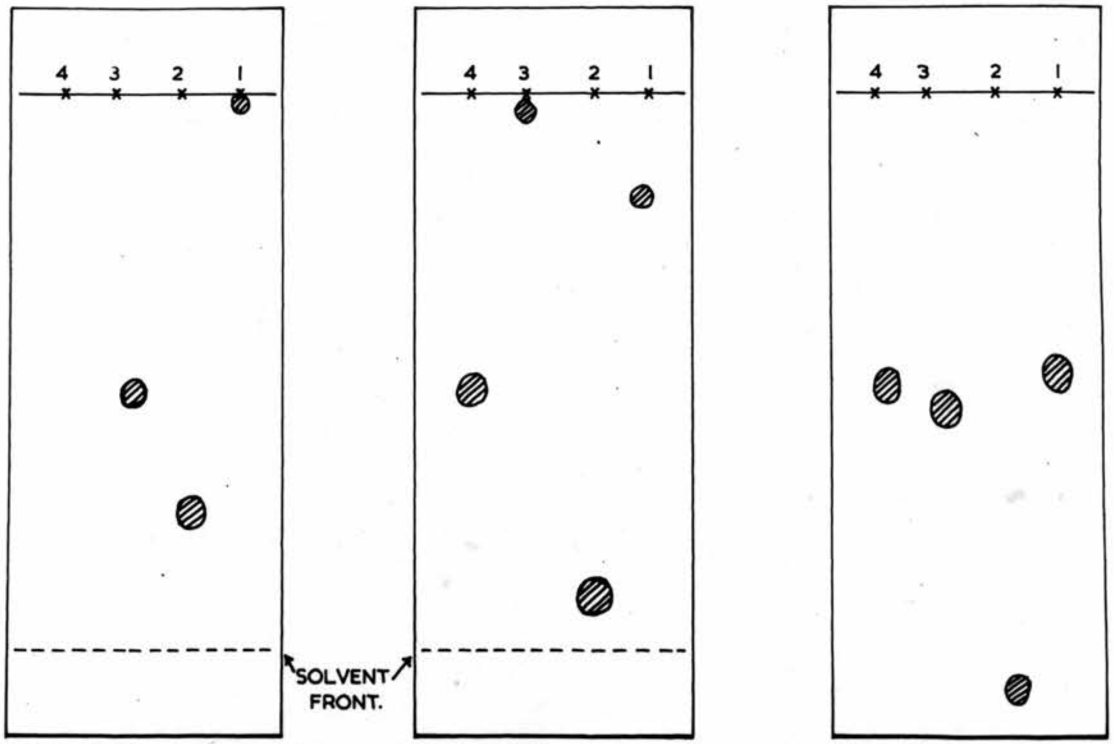
(a) 2-Methoxyoestriol - Fraction 4 yielded on removal of solvents 8.3 mg. yellow oil. According to the Kober reaction, there should be 1650 μg. Kober chromogen (calculated as 2-methoxyoestrone). Treatment of the oil with the common organic solvents failed to induce crystallization. The oil was finally dissolved in 4 ml. N-NaOH and saponified for 30 minutes. After cooling and diluting with 8.5% (w/v) $NaHCO_3$ (16 ml.) and water, the phenols were recovered by ether extraction. The phenolic residue obtained after removal/

FIG. 3, VII.

(a) SOLVENT SYSTEM:
70% MeOH in WATER/ BENZENE-HEXANE. (1:1, BY VOL.).

(b) SOLVENT SYSTEM:
CHLOROFORM-BENZENE (1:1, BY VOL.) / FORMAMIDE.

(c) SOLVENT SYSTEM:
CHLOROFORM/ $HCONH_2$



(a)
TIME OF RUN: 4·5 hrs.

1. CRUDE 2-METHOXYOESTRIOL.
2. 2-METHOXYOESTRONE.
3. OESTRONE.

(b)
TIME OF RUN: 4·5 hrs.

1. 16α-HYDROXYOESTRONE.
2. OESTRONE.
3. 2-METHOXYOESTRIOL.
4. OESTRADIOL-17β.

(c)
TIME OF RUN: 12 hrs.

1. 16-EPIOESTRIOL.
2. 16α-HYDROXYOESTRONE.
3. 17-EPIOESTRIOL.
4. CRUDE 2-METHOXYOESTRIOL.

removal of ether weighed 6 mg. However, despite this reduction in bulk, attempted crystallization of the oil was unsuccessful.

Fig. 3,VII shows the paper-grams obtained with crude 2-methoxyoestriol when run against standards in various solvent systems.

(b) <u>The 512.5 mμ Kober chromogen</u>

Fractions 7-9 afforded 6 mg. of oil on evaporation of solvents. This contained about 25% of Kober chromogen calculated as oestriol. Leaching of this material with a small volume of ethanol yielded 0.5 mg. of colourless solid which did not even melt at above 280°. The material recovered from the ethanol leaching solidified partially under ether. The semi-crystalline oil weighed 3.5 mg. Crystallization of this from chloroform containing a trace of methanol yielded 1.0 mg. of colourless solid. This proved to be the same material as was obtained earlier. It failed to melt at 300° and was negative in the Kober reaction.

The mother liquors were combined and evaporated to dryness. An aliquot of the oil, when chromatographed in the chloroform-formamide system/

system on paper for 12 hr. yielded a major spot indistinguishable from oestriol and a weak spot intermediate in 'polarity' between oestriol and 11β-hydroxyoestradiol-17β. In the David reaction an aliquot containing 50 μg. Kober chromogen gave a fairly strong green colour. Since 6β-hydroxyoestradiol-17β does not give the David reaction, it is concluded that the 512.5 mμ. Kober chromogen is oestriol.

7. Discussion

It appears from this preliminary study that the substance which gave an immediate red colour in the Kober reaction might not be related to the 512.5 mμ. Kober chromogens eventually detected in the urinary fraction after reduction with sodium borohydride. For, the intensity of the red colour produced suggested a much higher concentration than that found for the 512.5 mμ. Kober chromogen (*ca.* 2 mg./700 l. urine extract).

The nature of the original 'red chromogen' is not known. It may be a normal urinary constituent or a metabolite of some drug administered to the patients. For example, phenolphthalein/

phenolphthalein is known to give a red colour in the Kober reaction, which, however, does not fade away. Other drugs like cascara and senna are reported to interfere with the Kober reaction (cf. Marrian, 1956).

It has been shown that the more 'polar' 512.5 mμ Kober chromogen detected among the reduction products is oestriol, the origin of which is not without ambiguity. However, it seems likely that most of it must have been derived from small quantities of 16α-hydroxyoestrone which were present in fractions 13-14. If this were so, it follows that, rather unexpectedly, it was not resolved from 2-methoxy-KC-5 in alumina adsorption chromatography.

It is unlikely that much of the oestriol could have come from 16-oxooestrone, since reduction of micro quantities of pure 16-oxooestrone with sodium borohydride has been shown to yield over 90% of 16-*epi*oestriol and the remaining oestriol. On the other hand, 16-oxooestrone, if present in the fraction, would account for the presence of some of the less polar/

'polar'512.5 mμ. Kober chromogen. Though no investigations have been made, it seems likely that it contains at least some 16-*epi*oestriol.

These results do not preclude the other possibility that both 6-oxooestrone and 11-oxooestrone might have been present in the original fraction, albeit in very small amounts. Their reduction products, namely, 6β-hydroxyoestradiol-17β and 11β-hydroxyoestradiol-17β respectively, would not be resolved from oestriol (and, perhaps, 16-*epi*oestriol, too, in the case of 11β-hydroxyoestradiol-17β) in the system used. It is hoped to have this settled subsequently.

The presence of 2-methoxy-KC-5 in pregnancy urine was unexpectedly revealed in the course of this investigation. The failure of these methoxy derivatives to manifest themselves in the Kober reactions of the crude urine fraction is mainly due to (1) their relatively low concentration, (2) the interference of urinary contaminants in the Kober reaction, and (3) their low absorption at the Kober maximum.

The low concentration of the methoxy derivative/

derivatives in the final extract (ca. 3 mg./700 l. of urine) must be attributed to losses which, like those of KC-5, occur during the alkaline fraction and, perhaps, through destruction by ether peroxides. These become more serious when the excretion levels are lower. Moreover, it is not certain whether all the 2-methoxy-KC-5 was extracted from ether by alkali. If they were not,it is likely that quite a considerable portion would remain in the neutral fraction. No thorough search has yet been made for these compounds (and 2-methoxyoestrone, as well) in the neutral fraction.

However, even after making allowance for these, it still appears that the excretion of 2-methoxy-KC-5 is somewhat lower than that of 2-methoxyoestrone. This is in direct contrast to the finding that the excretion of KC-5 is about 3-4 times that of oestrone. Perhaps the explanation to this apparent anomaly lies at the biogenetic origin of 2-methoxy-KC-5 (or, more precisely, 2-methoxy-16α-hydroxyoestrone). This could possibly arise in two ways: by 16α-hydroxylation of 2-methoxyoestrone, or by methoxylation/

methoxylation of 16α-hydroxyoestrone. The latter reaction might presumably take place in the liver, and Schneider & Mason (1948) have demonstrated the oxidation of dehydro*epi*-androsterone at position 16 by rabbit liver.

The first metabolic pathway would seem to account for the lower excretion level observed for 2-methoxy-KC-5. However, it is possible that 16α-hydroxyoestrone, once conjugated in the liver, is rapidly eliminated from the body. For that matter, conjugation of the 3-phenolic group with either sulphuric or glucuronic acid would, at least on chemical grounds, inhibit hydroxylation at the ortho position (2 or 4). On the other hand, conjugation at C-16 might presumably not interfere with the methoxylation of ring A. Hence in consequence the amount of 2-methoxy-16α-hydroxyoestrone formed *via* the second route would be determined by the net effect of these various competing processes in the liver.

It is hoped, of course, to detect and eventually isolate 2-methoxyoestriol from pregnancy urine. However, if the first route were/

were the determinant factor in the production of 2-methoxy-16α-hydroxyoestrone, which subsequently yields on biological reduction 2-methoxyoestriol, it would not be surprising to find only small amounts of 2-methoxyoestriol in urine. It is believed that tracer technique would go a long way towards elucidating this interesting precursor-product relationship.

It is evident that new approaches must be made towards the isolation of 2-methoxy-16α-hydroxyoestrone. First and foremost is the necessity of preventing any rearrangement of the 16α-hydroxy-17-oxo to the 17β-hydroxy-16-oxo structure, which means avoiding the alkaline fractionation. The fractionation techniques used in the present study were not entirely satisfactory. For instance, though so useful in cleaning up the crude fraction, alumina adsorption chromatography has several disadvantages, among which are the difficulty of getting reproducibility and the possibility of destruction and/or rearrangement of the ketolic structure.
It/

It therefore appears that the use of paper chromatography would be of greater advantage not only in the isolation of 2-methoxy-16α-hydroxyoestrone, but also in the detection of minor oestrogen metabolites, which may be present in the same fraction, and which produce no or weak Kober colours.

APPENDIX I

Molecular Models of Oestrogen Derivatives

Molecular models have always been a useful adjunct in the elucidation of structural problems, while they are almost indispensable to the investigator engaged in the study of steric hindrance in organic compounds. Thus, with the detection of KC-6 in pregnancy urine, a series of studies on the molecular models of various oestrogen derivatives was initiated, the results of which are presented here.

Intramolecular hydrogen bonding

It is well known that hydrogen bonding is most effective when the distance between the bonding hydrogen and its oxygen counterpart, namely, the H -- O distance (cf. Kuhn, 1952) is a minimum. Thus, in examining the models of oestrogen derivatives for any indication of intramolecular hydrogen bonding the orientation of the hydroxyl group around the C-O bond must be made such that the H---O distance is a minimum. When/

When this is done with the four isomeric 16, 17-ketols, it will be observed that the H---O distance in 16α-hydroxyoestrone (and 16-oxo-oestradiol-17β) is small enough for hydrogen bonding to be effective. In the corresponding epimers of these two compounds, the H---O distances are much greater. Roughly speaking, the interatomic separations in 16α-hydroxy-oestrone (or 16-oxooestradiol-17β), 16-oxo-oestradiol-17α and 16β-hydroxyoestrone are in the proportion of 1:2:4.

Similarly, it can be deduced that intra-molecular hydrogen binding exists in both 16-_epi_-oestriol and 17-_epi_oestriol. On the other hand, the potential bonding groups in the trans glycols are separated too far apart for hydrogen bonding. Such a finding has already emerged from the infrared spectral studies on cyclic 1,2-diols by Kuhn (1954). It has been found that while an associated hydroxyl band is present in the solution (CCl_4) spectrum of cis-cyclopentane-1,2-diol, no such band is observed in that of the trans isomer. From the models it seems possible too/

too that this intramolecular hydrogen bonding is dimeric in nature.

Effect of intramolecular hydrogen bonding on chromatographic behaviour.

It seems reasonable to assume that when a hydroxyl group enters into hydrogen bonding its hydrophilic property would be partly neutralized. Likewise, the other functional group with which it is bonded, say, carbonyl or another hydroxyl, would lose to a certain degree its individual characteristic. Therefore, in chromatographic systems in which the extent of interaction (for instance, intermolecular hydrogen bonding) between the stationary phase and the dissolved solute determines its 'polarity', a substance with its functional groups masked by intramolecular hydrogen bonding, would possess a partition coefficient more in favour of the mobile phase than the stationary phase. Such considerations explain why 16-epioestriol is less 'polar' than oestriol, and why 16-epioestriol is not readily distinguishable from 17-epioestriol in several partition systems used (cf. Section III).

Moreover/

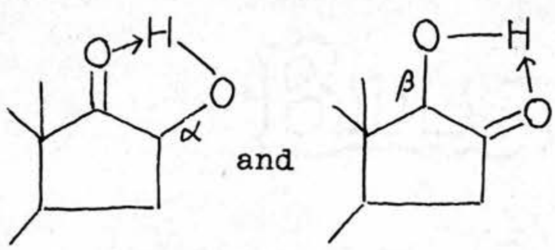
Moreover, the concept of hydrogen bonding might explain why when chromatographed together in the system 70% methanol in water/benzene-hexane (4:1, by vol.), the two _epi_oestriols moved rather like a 'unit', although, when run separately, their elution patterns were distinctly different, 17-_epi_oestriol being eluted some 6 to 8 ml. before 16-_epi_oestriol. In this case hydrogen bonding might also occur intermolecularly between 16-_epi_oestriol and 17-_epi_-oestriol, thus slowing down the 'less polar' one and hastening the 'polar' one. Incidentally, the shapes of these two molecules as judged by their models, seem to permit close approach to each other.

Nevertheless, resolution of the two _epi_-oestriols has been achieved by using the chloroform-formamide system as developed by Dr D.S. Layne. It is likely that in this Zaffaroni type of system adsorption as well as partition effects are called into play, thus resulting in a sharper differentiation of these two otherwise analogous structures.

It/

It may be expected that the unknown trans glycol, namely oestriol-16β:17α, would be similar to oestriol in chromatographic behaviour in most solvent systems.

The identical chromatographic behaviour of 16α-hydroxyoestrone and 16-oxooestradiol-17β in most partition systems is not surprising, since hydrogen bonding results in rather similar cyclic forms, viz.



In consequence the two functional groups do not exert their chromatographic properties individually.

On the other hand, similar considerations would lead to the anticipation that both 16β-hydroxyoestrone and 16-oxooestradiol-17α would be more 'polar' than their corresponding epimers in the same partition system. This has been shown to be so for 16β-hydroxyoestrone in the Bush type of system (cf. Section III B). More recently, Dr D.S. Layne obtained the same result with the chloroform/

chloroform-formamide system.

The chromatographic behaviour of other mono-hydroxy oestrones and oestradiols

The 18-hydroxyl can also enter into hydrogen bonding with the C-17 oxo group, as is indicated by the molecular model of 18-hydroxyoestrone. In this instance the conversion of the hydrophobic C-18 methyl to the hydrophilic hydroxymethyl group more than compensates for the decrease in 'polarity' due to the adverse bonding effect on the chromatographic mobility of 18-hydroxyoestrone. This might therefore account for the fact that KC-6A is more 'polar' than KC-5. Similarly, the 18-hydroxyl group can interact with the 17β-hydroxyl in 18-hydroxyoestradiol-17β. Perhaps, owing to the dimeric nature of such bonding, the latter compound is scarcely resolved from its precursor in the systems investigated so far. Thus, together with 16-*epi*oestriol and 17-*epi*oestriol, it belongs to the 'least polar' class of trihydroxy oestrogen derivatives.

The/

The finding that 11β-hydroxyoestradiol-17β is intermediate in 'polarity' between 16-<u>epi</u>-oestriol and oestriol seems to suggest that the location in the nucleus of the second alcoholic hydroxyl relative to the 3-phenolic hydroxyl and the 17β-hydroxyl is an important factor determining its chromatographic behaviour. Steric hindrance of the 11β-hydroxyl is now known to be not so serious in the oestrogens (cf. Magerlein & Hogg, 1957), and hence may not account entirely for the above finding. It is however possible that being located somewhat near to the centre of the molecule, the 11β-hydroxyl acts independently of the hydrophilic functions at the two ends of the molecule. In consequence it does not reinforce or weaken the influence of either of these hydroxyls. A 6β-hydroxyl, on the other hand, is much nearer to the 3-phenolic hydroxyl and each might conceivably enhance the effect of the other. This may be the reason why 6β-hydroxyoestradiol-17β is more 'polar' than the 11β-hydroxy derivative and is as 'polar' as oestriol. It is of/

of great interest to ascertain the effect of the disposition of the hydroxyl at C-7, 12 or 15 on chromographic mobility. This might then permit an evaluation of the validity of this simple postulate.

Intramolecular hydrogen bonding has been demonstrated in catechol but not in resorcinol (for example, see Kuhn, 1952), as is also indicated by means of the models. By analogy hydrogen bonding is to be expected in the oestrogen catechols. However, to what extent the mobility of 2- or 4-hydroxyoestrone is affected by hydrogen bonding, is less readily estimated, since electronic effects are called into play as well. It is therefore not certain in which fraction (KC-5 or KC-6) 2-hydroxyoestrone - the hypothetical metabolic precursor of 2-methoxyoestrone (Section VI) - would be found.

Steric hindrance in the oestrogen 16,17-ketols.

It has long been recognized that the course of reactions at a nuclear position of the steroid molecule is partly determined by its steric environment. This has been reviewed by Fieser in 1950 and with but a few exceptions the general rule/

rule of attack from the rear is amply illustrated therein.

The hindrance effect of the C-18 methyl group on the course of reactions at C-17 therefore requires no further elaboration. However, substitution at C-16 may also have an effect on the course of chemical events centred at C-17.

From an examination of the molecular models of the 16,17-ketols it might be expected that a 16α-hydroxyl would hinder to a certain extent the approach from the rear of a nucleophilic reagent (e.g. sodium borohydride) towards the C-17 oxo group, while a 16β-hydroxyl would be an additional obstacle to frontal attack. Consequently, chemical reduction of the C-17 oxo group would result in the formation of two epimers in the case of the 16α-substituted derivative, while the corresponding 16β-epimer would yield predominantly one product. Again, since in general catalytic effects are surface phenomena, steric requirements might well be more critical in catalytic hydrogenation than in reduction with chemical re-agents. The production of a higher proportion of the/

the 17α-epimer in the catalytic reduction of 16α-hydroxyoestrone might therefore be expected. By having a 16α-substituent larger than the C-18 methyl group the 'normal' course of reduction might conceivably be reversed, leading to the predominant formation of the 17α-epimer. These concepts, derived from purely mechanistic considerations, are in harmony with the findings described in Section III and the studies of other workers (e.g. Fajkos, 1955).

Hindrance effects around the C-16 oxo group are less marked. For one thing it is not within the immediate vicinity of the C-18 methyl group. However, with the introduction of a 17β-hydroxyl the steric environment around the C-16 oxo group might approximate to that around the C-17 oxo group in oestrone. Thus, attack from the rear by nucleophilic reagents would be favoured. The preferential opening of the rear bond of the C-16 carbonyl group would therefore lead to the predominant formation of the 16β-epimer. On the other hand, a 17α-hydroxyl might slightly impede the rear side approach of the reagent towards the C-16 carbonyl, and in consequence some 16α-epimer would also be produced. Thus, 16-oxooestradiol-17α on hydrogenation might yield some 17-<u>epi</u>-oestriol besides the main product, oestriol-16β:17α.

APPENDIX II

1. Oestrogen samples

Only those that were supplied to us by various generous donors will be mentioned. They are:-

(a) 6-oxooestradiol-17β diacetate, from Dr O. Wintersteiner

(b) 11β-hydroxyoestrone and 11β-hydroxyoestradiol-17β, from the Upjohn Company, Kalamazoo, Michigan, U.S.A.

(c) 2-methoxyoestrone, from Dr T.F. Gallagher

(d) 17-*epi*oestriol, from Dr L. Ruzicka.

The following were prepared on a micro scale from pure oestrogen derivatives and were used as chromatographic standards etc., without purification:-

(a) 6-oxooestradiol-17β, by acid hydrolysis of its diacetate

(b) 6β-hydroxyoestradiol-17β, by $NaBH_4$ reduction of 6-oxooestradiol-17β diacetate followed by acid hydrolysis

(c) 6β-hydroxyoestradiol-17β 3-methyl ether, prepared as described in (C), Methods, Section IV.

(d)/

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(d) 17-dihydroequilin, by alkaline hydrolysis of its 3-monobenzoate at room temperature.

2. Materials

The solvents and Celite (Celite 535, Johns-Manville Co. Ltd., London) used were purified by the methods employed by Watson & Marrian (1956). The chemicals used were mostly AR quality.

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16α-Hydroxyoestrone in the Urine of Pregnant Women

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Watson & Marrian (1955) detected the presence in extracts of the urine of pregnant women of a ketonic Kober chromogen which was more 'polar' than oestrone, and, on solvent-partition evidence, concluded that it was 16-oxo-oestradiol-17β (16-oxo-oestra-1:3:5-triene-3:17β-diol). More recently Marrian, Watson & Panattoni (1957) concentrated this Kober chromogen (KC-5) and showed conclusively that the major component in it must have been either 16α-hydroxyoestrone (17-oxo-oestra-1:3:5-triene-3:16α-diol) or 16β-hydroxyoestrone (17-oxo-oestra-1:3:5-triene-3:16β-diol), and not 16-oxo-oestradiol-17β. On biogenetic grounds the view was favoured that the major component in KC-5 was 16α-hydroxyoestrone rather than 16β-hydroxyoestrone, but since reduction with sodium borohydride yielded a mixture of oestriol (oestra-1:3:5-triene-3:16α:17β-triol) and 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) it was suggested that either 16β-hydroxyoestrone or artifactually produced 16-oxo-oestradiol-17β might also have been present in the isolated material.

For the further investigation of the nature of KC-5 it was clearly necessary to isolate a larger amount of more rigorously purified material from urine, and, since it seemed probable that the major component in the Kober chromogen might be 16α-hydroxyoestrone, it was also decided to attempt to prepare the latter by partial synthesis.

RESULTS

Preparation and properties of 16α-*hydroxyoestrone*

16α-Hydroxyoestrone diacetate, m.p. 172–174·5°, was prepared from oestrone by the method of Leeds, Fukushima & Gallagher (1954). These authors recorded m.p. 179–180° for their preparation. However, it would appear that the latter higher melting point is that of a different polymorphic modification of the same substance, since a sample of the preparation of Leeds *et al.* (1954) kindly supplied to us by Dr Gallagher was found to melt at 168·5–172°, and this melting point was not depressed after admixture with our own preparation. Moreover, Dr Gallagher has informed us that he has also observed a lower-melting modification of the substance.

After reduction of the diacetate by sodium borohydride, followed by alkaline hydrolysis, products were obtained which, as judged by partition-chromatographic analysis used in conjunction with the Kober reaction, appeared to contain about 81–85% of oestriol and about 15–19% of 16-*epi*-oestriol. It seems unlikely that the formation of this small proportion of a 16-*epi*oestriol-like product could have been due to the presence in the starting material of 16β-hydroxyoestrone diacetate or of 16-oxo-oestradiol-17β diacetate, both of which could be expected to yield mainly 16-*epi*oestriol on reduction by sodium borohydride followed by hydrolysis, since about the same relative amounts of the two products were formed irrespective of the number of times that the starting material had been recrystallized (Table 1). The possibility that 16-*epi*-oestriol was formed as a result of rearrangement of 16α-hydroxyoestrone diacetate to 16-oxo-oestradiol-17β diacetate during the reduction has not been excluded, but since the relative amounts of the two products could not be significantly altered by varying either the duration of the reaction or the amount of sodium borohydride used (Table 1) this explanation seems an unlikely one. A more probable explanation would seem to be that reduction of the 17-oxo group in 16α-hydroxyoestrone diacetate is less stereochemically specific for the formation of a 17β-hydroxyl group than it is in oestrone (cf. Biel, 1951), and that 17-*epi*oestriol (oestra-1:3:5-triene-3:16α:17α-triol) (Prelog, Ruzicka & Wieland, 1945), which is not clearly distinguishable from 16-*epi*oestriol in the solvent systems employed, was the 16-*epi*oestriol-like product which was formed.

Hydrolysis of 16α-hydroxyoestrone diacetate was effected somewhat as described by Leeds *et al.* (1954) for the rearrangement and hydrolysis of 16α:17α-epoxyandrostane-3β:17β-diol diacetate to 17-oxoandrostane-3β:16α-diol. After recrystallization the product melted at 238–240·5°, with marked shrinkage at about 216°, and had $[\alpha]_D^{16}+180°$ (ethanol). Acetylation of the hydrolysis product

Table 1. *Reduction of* 16α-*hydroxyoestrone diacetate by sodium borohydride in methanolic solution: relative proportions of oestriol and* '16-epi*oestriol' in the products after alkaline hydrolysis*

Wt. of 16α-hydroxy-oestrone diacetate (mg.)	Previous recrystallizations	Wt. of $NaBH_4$ (mg.)	Reduction time (min.)	Composition of hydrolysed reduction product (%)	
				Oestriol	'16-*epi*-Oestriol'
20·3	2 × ethyl acetate–*n*-hexane	15 + 10	40 + 60	81·2	18·8
20·0	2 × ethyl acetate–*n*-hexane 1 × benzene–*n*-hexane 3 × methanol	15 + 10	40 + 60	81·5	18·5
21·0	2 × ethyl acetate–*n*-hexane 1 × benzene–*n*-hexane 3 × methanol	5 + 2	15 + 20	83·5	16·5
20·0	2 × ethyl acetate–*n*-hexane 2 × methanol	4 + 2·5	15 + 20	81·2	18·8
20·0	2 × ethyl acetate–*n*-hexane 2 × methanol	2·5	35	82·2	17·8
20·0	2 × ethyl acetate–*n*-hexane 1 × benzene–*n*-hexane 5 × methanol	4 + 2	15 + 20	85·3	14·7
5·3	2 × ethyl acetate–*n*-hexane 2 × methanol	5	40	81·8	18·2

yielded an acetate, m.p. 169–173°, $[\alpha]_D^{20}+149$ (ethanol), and on admixture with 16α-hydroxyoestrone diacetate the melting point was not depressed. It was evident, therefore, that the hydrolysis product of the diacetate was indeed 16α-hydroxyoestrone.

16α-Hydroxyoestrone was also prepared more conveniently by treatment of 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate with methanolic sulphuric acid at room temperature, when rearrangement and hydrolysis occurred (cf. Leeds *et al.* 1954).

The melting-point behaviour of 16α-hydroxyoestrone requires special comment. The melting point, like that of the crude KC-5 isolated by Marrian *et al.* (1957), was not depressed after admixture with 16-oxo-oestradiol-17β (m.p. 239–241·5°), and this fact suggested that at or near its melting point 16α-hydroxyoestrone might undergo rearrangement to 16-oxo-oestradiol-17β. On redetermining the melting point of 16α-hydroxyoestrone with a more rapid rate of heating over the range 205–230° it was seen that the shrinkage previously observed at about 216° was in fact a fairly sharp melt, followed immediately by resolidification which was complete at about 222°. This suggested that the lower melting point might be the true one of 16α-hydroxyoestrone and that the higher one might be that of the hypothetical rearrangement product. In fact 16α-hydroxyoestrone was found to yield, when heated at 220–230° *in vacuo*, 16-oxo-oestradiol-17β, which was identified by its physical properties and those of its diacetate.

Reduction of 16α-hydroxyoestrone by sodium borohydride yielded a mixture containing about 90 and 10% respectively of oestriol-like and 16-*epi*-oestriol-like products. Accordingly, as with the diacetate, it would appear either that some rearrangement occurred during the reaction or that a small proportion of 17-*epi*oestriol was formed. As catalytic reduction with hydrogen and platinum oxide in neutral ethanolic solution yielded a mixture which contained a somewhat higher proportion (30%) of the '16-*epi*oestriol-like' product than was found after sodium borohydride reduction, the latter explanation seems the more probable one, since under these conditions it is unlikely that any rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β would have occurred.

On reduction with sodium borohydride the KC-5 isolated from urine by Marrian *et al.* (1957) yielded a relatively high proportion (45%) of 16-*epi*-oestriol, which was identified as such with certainty; and in explanation of this finding the possibility was considered, among others, that the isolated material might have consisted of a mixture of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β produced from the former by rearrangement during the extraction of the urinary phenolic fraction with aqueous sodium hydroxide. The fact that the urinary product had a significantly lower dextrorotation than that of 16α-hydroxyoestrone was clearly consistent with this possibility, and to investigate the latter further the behaviour of 16α-hydroxyoestrone in aqueous sodium hydroxide was studied.

In the preparation of the phenolic fraction of a urinary extract the time during which the fraction remains in alkaline solution is about 5–10 min.; accordingly, in the first instance, the effect on the optical rotation of 16α-hydroxyoestrone of allowing it to stand in aqueous N-sodium hydroxide for 10 min. at room temperature was examined. The phenolic product isolated from the reaction mixture showed a decrease in dextrorotation compared with that of the starting material which would have corresponded to the rearrangement of about 20% of the latter to 16-oxo-oestradiol-17β. That the observed decrease in dextrorotation was indeed due to partial rearrangement to 16-oxo-oestradiol-17β was shown by a second experiment in which 16α-hydroxyoestrone was treated with aqueous sodium hydroxide for 2 hr.: the product was identified as 16-oxo-oestradiol-17β by its physical properties and those of its diacetate. These findings support the view that the KC-5 isolated from pregnancy urine by Marrian *et al.* (1957) was a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxo-oestradiol-17β.

Isolation, purification and identification of KC-5

By use of the procedures described by Marrian *et al.* (1957) crude KC-5, $[\alpha]_D^{18}+114°$ (ethanol), was obtained from enzymically hydrolysed late-pregnancy urine. An attempt to purify directly the dextrorotatory component in this product by recrystallization from methanol failed, and accordingly it was decided to attempt purification via the acetate.

The acetylated product yielded two crops of apparently pure crystalline material: (*a*) m.p. 172·5–174·5°, $[\alpha]_D^{20}+146°$ (ethanol); and (*b*) m.p. 170–173°, $[\alpha]_D^{18}+157°$ (ethanol). Analysis of (*a*) gave C and H values in good agreement with those required for a compound of the formula $C_{22}H_{26}O_5$.

Conclusive proof of the identity of KC-5 acetate and 16α-hydroxyoestrone diacetate was provided as follows: (i) The melting point of product (*b*) was not depressed after admixture with 16α-hydroxyoestrone diacetate. (ii) The absorption spectra of solutions of KC-5 acetate (*b*) and 16α-hydroxyoestrone diacetate in concentrated sulphuric acid over the range 220–550 mμ. after 2 hr. at 25° were identical (Fig. 1). (iii) The infrared spectra of KC-5 acetate (*b*) and 16α-hydroxyoestrone diacetate, which were determined in potassium chloride disks by Dr R. K. Callow of the National Institute

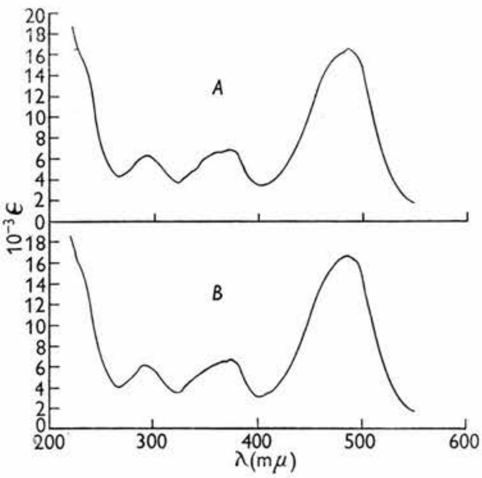

Fig. 1. Absorption spectra of H_2SO_4 solutions of (*A*) 16α-hydroxyoestrone diacetate and (*B*) KC-5 acetate after 2 hr. at 25°.

for Medical Research, 'showed complete similarity in all features, both in the main bands...and in all the bands in the "fingerprint" region...'.

Hydrolysis of KC-5 acetate with methanolic sulphuric acid at room temperature yielded a product which had $[\alpha]_D^{18}+169°$ (ethanol) and m.p. about 215° and 237·5–240°. The absorption spectrum of a solution of the product in concentrated sulphuric acid determined after 2 hr. closely resembled, but was not identical with, that of a similarly prepared solution of 16α-hydroxyoestrone. The slight differences between the absorption spectra and the fact that the optical rotation of the product was slightly low suggested that during the hydrolysis of KC-5 diacetate slight rearrangement had occurred. Unfortunately there was insufficient of the hydrolysis product to permit of more rigorous purification.

It is considered that completely satisfactory proof for the identity of KC-5 and 16α-hydroxyoestrone has been obtained.

EXPERIMENTAL

Methods

The procedures used for partition-chromatographic analysis, for the Kober reaction and for the determination of melting points, were as described by Marrian *et al.* (1957). All melting points are uncorrected for emergent stem.

Preparation and properties of 16α-hydroxyoestrone

16α-Hydroxyoestrone diacetate. This was prepared from 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate by treatment with perchloric and acetic acids as described by Leeds *et al.* (1954). The crude product of rearrangement was purified by filtration in benzene solution through a column of deactivated [10% (w/v) of water] Al_2O_3 and then by two crystallizations from ethyl acetate–*n*-hexane. The material had m.p. 169–172·5°, and partition-chromatographic analysis in the system 90% (v/v) ethanol in water/*n*-hexane did not reveal the presence of more than one Kober chromogen. Different portions of this material were further recrystallized from methanol and benzene–*n*-hexane. The portion for analysis was further recrystallized once from benzene–*n*-hexane and thrice from methanol, when it had m.p. 172–174·5°, $[\alpha]_D^{18}+126°$ (*c*, 0·502 in chloroform), $[\alpha]_D^{18}+153°$ (*c*, 0·503 in ethanol) (Found: C, 71·2; H, 6·8. Calc. for $C_{22}H_{26}O_5$: C, 71·3; H, 7·1%).

Reduction of 16α-hydroxyoestrone diacetate by sodium borohydride. The reduction of 16α-hydroxyoestrone diacetate by Na borohydride was studied in a series of experiments in which samples of the diacetate which had been recrystallized in different ways were allowed to react at room temperature in methanolic solution with varying amounts of the reductant for varying times. After hot alkaline hydrolysis the reaction mixtures were diluted with water, acidified with HCl and extracted with ether. The ether extracts were washed with 8·5% (w/v) $NaHCO_3$ and with water and evaporated to dryness. About 50 μg. of each product was analysed by partition chromatography, with the system 70% (v/v) methanol in water–ethylene dichloride, and in each case the presence of two different Kober chromogens chromatographically indistinguishable from oestriol and 16-*epi*oestriol respectively was detected. The more 'polar' Kober chromogen was isolated in one experiment and identified as oestriol by a mixed m.p. with an authentic specimen of the latter; in another experiment it was identified after acetylation by a mixed m.p. with authentic oestriol triacetate. Owing to lack of material conclusive evidence concerning the nature of the less 'polar' Kober chromogen was not obtained. The results of these experiments are summarized in Table 1.

Hydrolysis of 16α-hydroxyoestrone diacetate. To a solution of 170 mg. of 16α-hydroxyoestrone diacetate [m.p. 172–174·5°; $[\alpha]_D^{18}+150°$ (ethanol)] in 20 ml. of methanol was added 5 ml. of 5N-H_2SO_4 and the mixture allowed to stand at room temperature for 5 days. After dilution with 140 ml. of ethyl acetate the solution was washed with 8·5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product (130 mg.) on crystallization from methanol yielded 52 mg. of colourless crystals, m.p. about 216° and 238–240·5°, $[\alpha]_D^{16}+180°$ (*c*, 0·490 in ethanol) (Found: C, 75·6; H, 7·3. Calc. for $C_{18}H_{22}O_3$: C, 75·5; H, 7·7%).

Acetylation of a portion of this material with acetic anhydride and pyridine at room temperature overnight yielded a product which after crystallization from methanol had m.p. 169–173° and $[\alpha]_D^{20}+149°$ (*c*, 0·504 in ethanol). The melting point was not depressed after admixture either with the above-described 16α-hydroxyoestrone diacetate (m.p. 172–174·5°) or a sample of the latter (m.p. 168·5–172°) supplied by Dr T. F. Gallagher.

16α-Hydroxyoestrone from 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate. The epoxyacetate (45 mg.) was dissolved in a mixture of 4 ml. of methanol and 1 ml. of 5N-H_2SO_4, and the solution allowed to stand at room temperature for 5 days. After dilution with ethyl acetate the solution was washed with 85% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The product (37 mg.) yielded 11 mg. of colourless needles after crystallization from methanol. These had m.p.

in view of the finding that this compound readily undergoes rearrangement in alkaline solution to 16-oxo-oestradiol-17β there can be little doubt that the KC-5 previously isolated by Marrian *et al.* (1957) was indeed a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxo-oestradiol-17β as was suggested. The available evidence strongly suggests that extracts of late-pregnancy urine contain no 16-oxo-oestradiol-17β other than that which must be artifactually formed from 16α-hydroxyoestrone during the extraction of the phenolic fraction by aqueous alkali from ether. In view of this the recent claim by Levitz, Spitzer & Twombly (1956) to have detected the presence of radioactive 16-oxo-oestradiol-17β in the urine of human subjects after the administration of [16-^{14}C]oestradiol-17β must be treated with reserve.

The isolation of 16α-hydroxyoestrone from urine lends support to the previous suggestion of Marrian *et al.* (1957) that this compound may be formed from oestrone by 16α-hydroxylation and may be the metabolic intermediate in the 'hydration' of oestrone to oestriol.

SUMMARY

1. 16α-Hydroxyoestrone diacetate, prepared from 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate by the method of Leeds *et al.* (1954), yielded 16α-hydroxyoestrone on cold acid hydrolysis. 16α-Hydroxyoestrone was also prepared directly from the epoxyacetate by cold acid treatment.

2. 16α-Hydroxyoestrone readily undergoes rearrangement to 16-oxo-oestradiol-17β in aqueous alkali at room temperature. The same rearrangement occurs when it is heated *in vacuo* to 220–230°.

3. A preparation of the ketonic-phenolic Kober chromogen (KC-5) prepared from enzymically-hydrolysed late-pregnancy urine as described by Marrian *et al.* (1957) yielded a product after acetylation which has been conclusively identified as 16α-hydroxyoestrone diacetate.

4. In view of the rearrangement undergone by 16α-hydroxyoestrone in alkaline solution it is concluded that KC-5 as isolated from urine by the methods described consists of 16α-hydroxyoestrone contaminated with about 20% of artifactually produced 16-oxo-oestradiol-17β.

5. It is suggested that 16α-hydroxyoestrone may be the metabolic intermediate between oestrone and oestriol.

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The isolation of a sixth Kober chromogen from the urine of pregnant women and its provisional identification as 18-hydroxyoestrone

By partition chromatography on Celite columns with the solvent system 70% (v/v) methanol in water–20% (v/v) *n*-hexane in benzene, a Kober chromogen (KC-6) more "polar" than 16α-hydroxyoestrone was detected in ketonic–phenolic fractions obtained from the urine of pregnant women as described by MARRIAN, WATSON AND PANATTONI[1] and MARRIAN, LOKE, WATSON AND PANATTONI[2]. Treatment of concentrates of KC-6, prepared from such fractions by partition chromatography, with small volumes of chilled chloroform yielded a nearly white, sparingly soluble, solid (KC-6A) in yields of about 2–3 mg/100 l urine, and a soluble fraction, believed to contain another Kober chromogen (KC-6B), which, however, has not yet been isolated.

Purification of KC-6A by further leaching with chilled chloroform, crystallization from methanol-benzene and from ethanol yielded white crystals, m.p. 255–257° (uncorr.; evac. sealed capillary), $[\alpha]_D^{19} + 146°$ (ethanol). (Found: C, 75.4; H, 7.9. Calc. for $C_{18}H_{22}O_3$: C, 75.5; H, 7.7).

In the Kober reaction of BROWN[3] as modified by BAULD[4], using the latter's "oestriol reagent", KC-6A gave a pink colour, the optical density of which at 512.5 mμ was only about 20% of that given by an equal amount of oestriol under the same conditions. In the "blue tetrazolium" test of MADER AND BUCK[5], KC-6A showed negligible reducing power, indicating that it is not an α-ketol.

On reduction with sodium borohydride in methanolic solution KC-6A yielded a non-ketonic product (Girard reaction), which had the same R_F as KC-6A when chromatographed on paper in the solvent system: benzene, 70; chloroform, 40; methanol, 70; water, 35. The optical density at 512.5 mμ of the pink colour given in the Kober reaction by this reduction product was about 130% greater than that given by an equal amount of KC-6A under the same conditions.

The infrared spectrum of KC-6A (KCl disc) showed no band at 1377 cm^{-1}. Since in other steroids a band at this wave number has been ascribed to the methyl group attached to C-13 (JONES AND COLE[6]; JONES, COLE AND NOLIN[7]), this finding suggested the possibility that in KC-6A the group attached to C-13 might have an oxygen substituent. After treatment of KC-6A with *N*–NaOH at room temperature for 4 h, 0.90 molar equivalents of formaldehyde (determined by the chromotropic acid reaction) were evolved on acidification and distillation; and a non-volatile, ketonic–phenolic product was obtained which gave no colour in the Kober reaction, but which had the same R_F as oestrone when chromatographed on paper in the solvent system: benzene, 50; *n*-hexane, 50; methanol, 70; water, 30. By analogy with the action of alkali on methyl hederagonate and on icterogenin (BARTON AND DE MAYO[8]), it seems probable that KC-6A possesses a primary β-ketol grouping. Hence it is provisionally concluded that KC-6A is 18-hydroxyoestrone, and that the non-volatile ketonic–phenolic product formed from it on alkali treatment is 18-noroestrone.

Since KAHNT, NEHER AND WETTSTEIN[9] have shown that 11-deoxycorticosterone becomes hydroxylated at C-18 and C-19 on incubation with ox-adrenal homogenates, an attempt was made to demonstrate the formation of KC-6A from oestrone under the same conditions. In several experiments the formation of about 0.2% of a Kober chromogen indistinguishable from KC-6A in its partition chromatographic behaviour was demonstrated. Furthermore, on treatment with alkali the chromatographically purified Kober chromogen yielded formaldehyde in about the expected amount and a non-volatile product was formed which was indistinguishable by paper chromatography from the alkali degradation product of KC-6A. These findings are compatible with the view that KC-6A is 18-hydroxyoestrone, and that it is a metabolic product of oestrone that is formed in the adrenal glands.

Full experimental details of this work will be published elsewhere.

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Isolation of 2-methoxyoèstrone from the urine of pregnant women

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Isolation of 2-methoxyoestrone from the urine of pregnant women

KRAYCHY AND GALLAGHER[1] have recently reported the isolation of 2-methoxyoestrone from the urine of human subjects following the administration of oestradiol-17β-16 ^{14}C. This finding has been confirmed by ENGEL *et al.*[2]. In the course of our own studies on urinary oestrogen metabolites, the same compound has now been isolated from the urine of pregnant women, and observations of some interest have been made concerning its behaviour in the Kober reaction.

Preliminary experiments were carried out on synthetic 2-methoxyoestrone generously supplied by Dr. T. F. GALLAGHER of the Sloan-Kettering Institute, New York.

In the Kober reaction carried out by the procedure of BROWN[3] as modified by BAULD[4], using the latter's "oestriol reagent", 2-methoxyoestrone gave an immediate orange-pink colour in the first stage of the reaction which changed to red after 20 min heating. In the second stage of the reaction the colour changed to a purple-pink, which showed a rather flat absorption maximum between 545 and 550 mμ (*cf.* oestriol and oestrone, max. at 512.5 mμ).

Good separation of 2-methoxyoestrone from oestrone was achieved by chromatography on a Celite column using the solvent system 70% (v/v) methanol in water/30% (v/v) benzene in *n*-hexane.

The starting material for the isolation was ketonic-phenolic material from 700 l of enzymically hydrolysed late pregnancy urine prepared as described by MARRIAN *et al.*[5]. The fraction of this containing oestrone and less "polar" ketonic phenols had been previously separated on a Celite column in the system 70% (v/v) methanol in water/20% (v/v) *n*-hexane in benzene. This fraction was chromatographed on Celite using the system 70% (v/v) methanol in water/30% (v/v) benzene in *n*-hexane. Those portions of the eluate which gave a Kober reaction suggestive of the presence of 2-methoxyoestrone were combined and yielded 94 mg of a brown oil. On leaching this with a small volume of acetone at —20° about 10 mg of a white crystalline substance was obtained. This gave a negative Kober reaction and was not further investigated. The material soluble in cold acetone was chromatographed on Al_2O_3 (acid washed; activated by heating at 140°) using benzene-hexane, benzene and acetone-benzene for elution. The fractions richest in the 2-methoxyoestrone-like Kober chromogen were combined and recrystallized twice from methanol at —20°, when 2.1 mg of a white crystalline product, m.p. 183–184° (uncorr.), was obtained. A further 2.1 mg of material, m.p. 182–183°, was obtained from the mother liquors. The infrared spectrum on a sample of the product was kindly determined by Dr. T. F. GALLAGHER who reported that it was "identical in all respects with that of 2-methoxyoestrone".

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